This Assignment 1000 is in the ENGLISH language and has the same meaning in other languages with the same 1000 number and R/Date.

# DECLARATION AND POWER OF ATTORNEY

USA/PCT

As a below named inventor, I hereby declare that:

(a) My residence and Citizenship are as stated below my name. My P.O. (mailing) address is the same as my residence unless otherwise stated.

(b) I verily believe I am/we are the original, first and sole/joint inventor(s) of the subject matter which is embraced by and for which a patent is sought on the invention entitled: **HUMANIZED ANTI-TAG-72 MONOCLONAL ANTIBODIES USING HUMAN SUBGROUP 4 KAPPA LIGHT CHAINS**

and the specification of which: (check one) ☐ is attached hereto (_____).
☒ was filed on October 30, 1997 as (37777E).
Application No. 08/961,309
and was amended on _____

(c) I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

(d) I acknowledge my duty under 37 CFR 1.56 to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in 37 CFR 1.56(b). I acknowledge the same duty of disclosure with respect to information which is first published or which arises after the filing date of any prior application claimed under paragraph (f) to the extent that the subject matter of any claim of this application is not disclosed in the prior United States application.

(e) I hereby claim foreign priority benefits under Title 35, United States Code § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

| PRIOR FOREIGN APPLICATION(S) | | | PRIORITY CLAIMED |
|---|---|---|---|
| Number | Country | Day/Month/Year Filed | ☐ YES ☐ NO |

(f) I hereby Claim the benefit under 35 U.S.C. §119 of any United States provisional application(s) or §120 of any United States application(s) listed below:

| | | |
|---|---|---|
| **07/510,697** | **07/17/90** | **Abandoned** |
| **07/964,536** | **10/20/92** | **Abandoned** |
| **08/261,354** | **06/16/94** | **Pending** |
| **60/030,173** | **10/31/96** | **Abandoned** |
| Application Serial No. | Filing Date | Status (Patented, Pending, Abandoned) |

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. Address all correspondence to: **STEPHEN S. GRACE at P.O. BOX 1967, MIDLAND, MICHIGAN 48641-1967** and telephonic communications to the following:

| | | |
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This appointment, including the right to delegate this appointment, shall also apply to the same extent to any proceedings established by the Patent Cooperation Treaty.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Title 18, United States Code § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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( ☒ ) Additional names and signatures are attached.

Docket Ref.: 37777E

Additional names, addresses and signatures to be attached to Form No. 1000

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# COMPOSITE ANTIBODIES OF HUMANIZED HUMAN SUBGROUP IV LIGHT CHAIN CAPABLE OF BINDING TO TAG-72

## Cross-Reference to Related Application

The Applicants herein claim the benefit of priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 60/030,173 (Attorney Docket No. 41,290) entitled, "Humanized Monoclonal Antibodies Specific to TAG-72, Methods for Their Manufacture and Usage in the Treatment or Diagnosis of Cancer," which was filed on October 31, 1996 by W.H. Kerr Anderson *et al*. The present application is a Continuation-in-Part of copending Application Serial Number 08/261,354, which is a Continuation-in-Part of Application Serial Numbers 07/510,697, filed 7/17/90, and 07/964,536, filed 10/20/92, both now abandoned.

## Field of the Invention

The present invention is directed to the fields of immunology and genetic engineering.

## Background of the Invention

The following information is provided for the purpose of making known information believed by the applicants to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the following information constitutes prior art against the present invention.

Antibodies are specific immunoglobulin (Ig) polypeptides produced by the vertebrate immune system in response to challenges by foreign proteins, glyco-proteins, cells, or other antigenic foreign substances. The binding specificity of such polypeptides to a particular antigen is highly

refined, with each antibody being almost exclusively directed to the particular antigen which elicited it.

Two major methods of generating vertebrate antibodies are presently utilized: generation *in situ* by the mammalian B lymphocytes and generation in cell culture by B cell hybrids. Antibodies are generated *in situ* as a result of the differentiation of immature B lymphocytes into plasma cells (see Gough (1981), Trends in Biochem Sci, **6**:203). Even when only a single antigen is introduced into the immune system of a particular mammal, a uniform population of antibodies does not result, i.e., the response is polyclonal.

The limited but inherent heterogeneity of polyclonal antibodies is overcome by the use of hybridoma technology to create "monoclonal" antibodies in cell cultures by B cell hybridomas (see Kohler and Milstein (1975), Nature, **256**:495--497). In this process, a mammal is injected with an antigen, and its relatively short-lived, or mortal, splenocytes or lymphocytes are fused with an immortal tumor cell line. The fusion produces hybrid cells or "hybridomas" which are both immortal and capable of producing the genetically-coded antibody of the B cell.

In many applications, the use of monoclonal antibodies produced in non-human animals is severely restricted where the monoclonal antibodies are to be used in humans. Repeated injections in humans of a "foreign" antibody, such as a mouse antibody, may lead to harmful hypersensitivity reactions, i.e., an anti-idiotypic, or anti-mouse antibody (HAMA), response (see Shawler *et al.* (1985), Journal of Immunology, **135**:1530-1535; and Sear *et al.*, J. Biol. Resp. Modifiers, **3**:138-150).

Various attempts have already been made to manufacture human-derived monoclonal antibodies by using human hybridomas (see Olsson *et al.* (1980), Proc. Natl. Acad. Sci. USA, **77**:5429; and Roder *et al.* (1986), Methods in Enzymology,

**121**:140-167). Unfortunately, yields of monoclonal antibodies from human hybridoma cell lines are relatively low compared to mouse hybridomas. In addition, human cell lines expressing immunoglobulins are relatively unstable compared to mouse cell lines, and the antibody producing capability of these human cell lines is transient. Thus, while human immunoglobulins are highly desirable, human hybridoma techniques have not yet reached the stage where human monoclonal antibodies with required antigenic specificities can be easily obtained.

Thus, antibodies of nonhuman origin have been genetically engineered to create chimeric or humanized antibodies. Such genetic engineering results in antibodies with a reduced risk of a HAMA response compared to that expected after injection of a human patient with a mouse antibody. In a chimeric antibody, non-human regions of immunoglobulin constant sequences are replaced by corresponding human ones (see USP 4,816,567 to Cabilly *et al.*, Genentech); in a humanized antibody, complementarity determining regions (CDRs) are grafted onto human framework regions (FR) (see European Patent Office Application (EPO) 0 239 400 to Winter). Some researchers have produced Fv antibodies (see USP 4,642,334 to Moore, DNAX) and single chain Fv (SCFV) antibodies (see USP 4,946,778 to Ladner, Genex).

The above patent publications only show the production of antibody fragments in which some portion of the variable domains is coded for by nonhuman V gene regions. Humanized antibodies to date still retain various portions of light and heavy chain variable regions of nonhuman origin: the chimeric, Fv and single chain Fv antibodies retain the entire variable region of nonhuman origin and CDR-grafted antibodies retain CDR of nonhuman origin.

Such nonhuman-derived regions are expected to elicit an immunogenic reaction when administered into a human patient (see Brüggemann *et al.* (1989), J. Exp. Med., **170**:2153-2157; and Lo Buglio (1991), Sixth International Conference on

Monoclonal Antibody Immunoconjugates for Cancer, San Diego, Ca). Thus, it is most desirable to obtain a human variable region which is capable of binding to a selected antigen.

One known human carcinoma tumor antigen is tumor-associated glycoprotein-72 (TAG-72), as defined by monoclonal antibody B72.3 (see Thor *et al.* (1986) Cancer Res., **46**:3118-3124; and Johnson, *et al.* (1986), Cancer Res., **46**:850-857). TAG-72 is associated with the surface of certain tumor cells of human origin, specifically the LS174T tumor cell line (American Type Culture Collection (ATCC) No. CL 188), which is a variant of the LS180 (ATCC No. CL 187) colon adeno-carcinoma line.

Numerous murine monoclonal antibodies have been developed which have binding specificity for TAG-72. Exemplary murine monoclonal antibodies include the "CC" (colon cancer) monoclonal antibodies, which are a library of murine monoclonal antibodies developed using TAG-72 purified on an immunoaffinity column with an immobilized anti-TAG-72 antibody, B72.3 (ATCC HB-8108) (see EP 394277, to Schlom *et al.*, National Cancer Institute). Certain CC antibodies were deposited with the ATCC: CC49 (ATCC No. HB 9459); CC83 (ATCC No. HB 9453); CC46 (ATCC No. HB 9458); CC92 (ATCC No. HB 9454); CC30 (ATCC NO. HB 9457); CC11 (ATCC No. 9455) and CC15 (ATCC No. HB 9460). Various antibodies of the CC series have been chimerized (see, for example, EPO 0 365 997 to Mezes *et al.*, The Dow Chemical Company).

It is thus of great interest to develop antibodies against TAG-72 containing a light and/or heavy chain variable region(s) derived from human antibodies. However, the prior art simply does not teach recombinant and immunologic techniques capable of routinely producing an anti-TAG-72 antibody in which the light chain and/or the heavy chain variable regions have specificity and affinity for TAG-72 and which are derived from human sequences so as to elicit expectedly low or no HAMA response. It is known that the

function of an immunoglobulin molecule is dependent on its three dimensional structure, which in turn is dependent on its primary amino acid sequence. A change of a few or even one amino acid can drastically affect the binding function of the antibody, i.e., the resultant antibodies are generally presumed to be a non-specific immunoglobulin (NSI), i.e., lacking in antibody character, (see, for example, USP 4,816,567 to Cabilly *et al.*, Genentech).

Surprisingly, the present invention is capable of meeting many of these above mentioned needs and provides a method for supplying the desired antibodies. For example, in one aspect, the present invention provides a cell capable of expressing a composite antibody having binding specificity for TAG-72, said cell being transformed with (a) a DNA sequence encoding at least a portion of a light chain variable region ($V_L$) effectively homologous to the human Subgroup IV germline gene (Hum4 $V_L$), and a DNA sequence segment encoding at least a portion of a heavy chain variable region ($V_H$) capable of combining with the $V_L$ into a three dimensional structure having the ability to bind to TAG-72.

In one aspect, the present invention concerns a composite antibody or antibody fragment comprising a DNA sequence encoding at least one chain which comprises a variable region having a heavy chain ($V_H$) and a light chain ($V_L$), (A) said $V_H$ being encoded by a DNA sequence comprising a subsegment effectively homologous to the $V_H\alpha$TAG germline gene ($V_H\alpha$TAG), and (B) said $V_L$ being encoded by a DNA sequence comprising a subsegment effectively homologous to the human Subgroup IV germline gene ($Hum_kIV$).

In another aspect, the present invention provides a composite antibody or antibody fragment having binding specificity for TAG-72, comprising (a) a DNA sequence encoding at least a portion of a light chain variable region ($V_L$) effectively homologous to the human Subgroup IV germline gene (Hum4 $V_L$), and a DNA sequence segment encoding at least a

portion of a heavy chain variable region ($V_H$) capable of combining with the $V_L$ into a three dimensional structure having the ability to bind TAG-72.

The invention further includes the aforementioned antibody alone or conjugated to an imaging marker or therapeutic agent. The invention also includes a composition comprising the aforementioned antibody in unconjugated or conjugated form in a pharmaceutically acceptable, non-toxic, sterile carrier.

The invention is also directed to a method for *in vivo* diagnosis of cancer which comprises administering to an animal containing a tumor expressing TAG-72 a pharmaceutically effective amount of the aforementioned composition for the *in situ* detection of carcinoma lesions.

The invention is also directed to a method for intraoperative therapy which comprises (a) administering to a patient containing a tumor expressing TAG-72 a pharmaceutically effective amount of the aforementioned composition, whereby the tumor is localized, and (b) excising the localized tumors.

Additionally, the invention also concerns a process for preparing and expressing a composite antibody. Some of these processes are as follows. A process which comprises transforming a cell with a DNA sequence encoding at least a portion of a light chain variable region ($V_L$) effectively homologous to the human Subgroup IV germline gene (Hum4 $V_L$), and a DNA sequence segment encoding at least a portion of a heavy chain variable region ($V_H$) which is capable of combining with the $V_L$ to form a three dimensional structure having the ability to bind to TAG-72. A process for preparing a composite antibody or antibody which comprises culturing a cell containing a DNA sequence encoding at least a portion of a light chain variable region ($V_L$) effectively homologous to the human Subgroup IV germline gene (Hum4 $V_L$), and a DNA sequence segment encoding at least a portion of a heavy chain

variable region ($V_H$) capable of combining with the $V_L$ into a three dimensional structure having the ability to bind to TAG-72 under sufficient conditions for the cell to express the immunoglobulin light chain and immuno-globulin heavy chain. A process for preparing an antibody conjugate comprising contacting the aforementioned antibody or antibody with an imaging marker or therapeutic agent.

Description of the Drawings

Figure 1 illustrates a basic immunoglobulin structure.

Figure 2 illustrates the nucleotide sequences of $V_H\alpha$TAG, CC46 $V_H$, CC49 $V_H$, CC83 $V_H$ and CC92 $V_H$.

Figure 3 illustrates the amino acid sequences of $V_H\alpha$TAG, CC46 $V_H$, CC49 $V_H$, CC83 $V_H$ and CC92 $V_H$.

Figure 4 illustrates the $V_H$ nucleotide and amino acid sequences of antibody B17X2.

Figure 5 illustrates the mouse germline J-H genes from pNP9.

Figure 6 illustrates the plasmid map of p49 g1-2.3.

Figure 7 illustrates the plasmid map of p83 g1-2.3.

Figure 8 illustrates the entire sequence of HUMVL(+) and HUMVL(-).

Figure 9 illustrates the human J4 (HJ4) nucleotide sequence and amino acid sequence.

Figure 10 illustrates the nucleotide sequences, and the amino acid sequences of Hum4 $V_L$, *ClaI-Hin*dIII segment.

Figure 11 illustrates a schematic representation of the human germline Subgroup IV $V_L$ gene (Hum4 $V_L$), as the target for the PCR.

Figure 12 shows the results of an agarose gel electrophoresis of a PCR reaction to obtain the Hum4 $V_L$ gene.

Figure 13 illustrates the restriction enzyme maps of pRL1000, and precursor plasmids pSV2neo, pSV2neo-101 and pSV2neo-102. "X" indicates where the *Hin*dIII site of pSV2neo has been destroyed.

Figure 14 illustrates a polylinker segment made by synthesizing two oligonucleotides: CH(+) and CH(-).

Figure 15 illustrates a primer, NEO102SEQ, used for sequencing plasmid DNA from several clones of pSV2neo-102.

Figure 16 illustrates an autoradiogram depicting the DNA sequence of the polylinker region in pSV2neo-102.

Figure 17 illustrates a partial nucleotide sequence segment of pRL1000.

Figure 18 illustrates the restriction enzyme map of pRL1001.

Figure 19 illustrates an autoradiogram of DNA sequence for pRL1001 clones.

Figure 20 illustrates a competition assay for binding to TAG-using a composite Hum4 $V_L$, $V_H\alpha$TAG antibody.

Figure 21 illustrates a general DNA construction of a single chain, composite Hum4 $V_L$, $V_H\alpha$TAG.

Figure 22 illustrates the nucleotide sequence and amino acid sequence of SCFV1.

Figure 23 shows the construction of plasmid pCGS515/SCFV1.

Figure 24 shows the construction of plasmid pSCFV31.

Figure 25 shows the construction of *E. coli* SCFV expression plasmids containing Hum4 $V_L$.

Figure 26 shows the DNA sequence and amino acid sequence of Hum4 $V_L$-CC49$V_H$ SCFV present in pSCFVUHH.

Figure 27 shows the construction plasmid pSCFV UHH and a schematic of a combinatorial library of $V_H$ genes with Hum4 $V_L$.

Figure 28 illustrates the nucleotide sequence of FLAG peptide adapter in pATDFLAG.

Figure 29 illustrates the construction of pATDFLAG, pHumVL-HumVH (X) and pSC49FLAG.

Figure 30 illustrates the nucleotide and amino acid sequences of pSC49FLAG.

Figure 31 shows the flow diagram for the discovery of HUM4 $V_L$-$V_H$ combinations that compete with prototype TAG-binding antibodies or mimetics.

Figure 32 illustrates the "humanization" protocols used in Example 6 to produce the humanized antibody variable regions derived from CC49.

Figure 33 illustrates the nucleotide sequences of the humanized CC49 (HuCC49*) variable regions genes.

Figure 34 is a schematic illustration of the process used in Example 6 to form the eukaryotic expression constructs of the humanized light (A) and heavy (B) chains of HuCC49*.

Figure 35 illustrates SDS-PAGE analyses of purified HuCC49* and cCC49 under non-reducing (A) and reducing (B) conditions.

Figure 36 illustrates HPCL analyses of (A) radioiodinated HuCC49* ($^{131}$I-labeled) and (B) radioiodinated cCC49 ($^{125}$I-labeled) MAbs.

Figure 37 shows the reactivity of HuCC49*, cCC49, and nCC49 in a competition RIA against $^{125}$I-labeled nCC49 bound to BSM-immobilized TAG-72.

Figure 38 shows the clearance of radioiodinated HuCC49* and cCC49 MAbs from the serum of mice.

## Detailed Description of the Invention

Prior to setting forth the invention, definitions of certain terms which are used in this disclosure are set forth below:

Antibody - This refers to single chain, two-chain, and multi-chain proteins and glycoproteins belonging to the classes of polyclonal, monoclonal, chimeric, and hetero immunoglobulins (monoclonal antibodies being preferred); it also includes synthetic and genetically engineered variants of these immunoglobulins. "Antibody fragment" includes Fab, Fab', $F(ab')_2$, and Fv fragments, as well as any portion of an antibody having specificity toward a desired target epitope or epitopes.

Humanized antibody - This will refer to an antibody derived from a non-human antibody, typically murine, that retains or substantially retains the antigen-binding properties of the parent antibody but which is less immunogenic in humans. This may be achieved by various methods including (a) grafting only the non-human CDRs onto human framework and constant regions with or without retention of critical framework residues, or (b) transplanting the entire non-human variable domains, but "cloaking" them with a human-like section by replacement of surface residues. Such methods as are useful in practicing the present invention include those disclosed in Jones et al., Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984); Morrison and Oi, *Adv. Immunol.*, 44:65-92 (1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988); Padlan, *Molec. Immun.*, 28:489-498 (1991); Padlan, *Molec. Immun.*, 31(3):169-217 (1994).

Complementarity Determining Region, or CDR - The term CDR, as used herein, refers to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site as delineated by Kabat et al (1991).

Framework Region - The term FR, as used herein, refers to amino acid sequences interposed between CDRs. These portions of the antibody serve to hold the CDRs in an appropriate orientation for antigen binding.

Constant Region - The portion of the antibody molecule which confers effector functions. In the present invention, murine constant regions are substituted with human constant regions. The constant regions of the subject chimeric or humanized antibodies are derived from human immunoglobulins. The heavy chain constant region can be selected from any of the five isotypes: alpha, delta, epsilon, gamma or mu. Further, heavy chains of various subclasses (such as the IgG subclasses of heavy chains) are responsible for different effector functions and thus, by choosing the desired heavy chain constant region chimeric antibodies with desired effector function can be produced. Preferred constant regions are gamma 1 (IgG1), gamma 3 (IgG3) and gamma 4 (IgG4). More preferred is a constant region of the gamma 1 (IgG1) isotype. The light chain constant region can be of the kappa or lambda type, preferably of the kappa type.

Chimeric antibody - This is an antibody containing sequences derived from two different antibodies, which typically are of different species. Most typically chimeric antibodies comprise human and murine antibody fragments, generally human constant and murine variable regions.

Mammals - Animals that nourish their young with milk secreted by mammary glands, preferably warm blooded mammals, more preferably humans.

Immunogenicity - A measure of the ability of a targeting protein or therapeutic moiety to elicit an immune response (humoral or cellular) when administered to a recipient. The present invention is concerned with the immunogenicity of the subject humanized antibodies or fragments thereof.

Humanized antibody of reduced immunogenicity - This refers to a humanized antibody exhibiting reduced immunogenicity relative to the parent antibody.

Humanized antibody substantially retaining the binding properties of the parent antibody - This refers to a humanized antibody which retains the ability to specifically bind the

antigen recognized by the parent antibody used to produce such humanized antibodies. Preferably the humanized antibody will exhibit the same or substantially the same antigen-binding affinity and avidity as the parent antibody, e.g., CC49. Preferably, the affinity of the antibody will at least about 10% of that of the parent antibody. More preferably, the affinity will be at least about 25%, i.e. at least two-fold less than the affinity of the parent antibody. Most preferably the affinity will be at least about 50% that of the parent antibody. Methods for assaying antigen-binding affinity are well known in the art and include half-maximal binding assays, competition assays, and Scatchard analysis. Suitable antigen binding assays are described in this application.

In a preferred embodiment, the antibodies and fragments of the present invention will be substantially homologous with those exemplified below and/or presented in the Figures. The phrase "substantially homologous" is used in regard to the similarity of a subject amino acid sequence (of an oligo- or poly-peptide or protein) to a related, reference amino acid sequence. This phrase is defined as at least about 75% "correspondence" -- i.e. the state of identical amino acid residues being situated in parallel -- between the subject and reference sequences when those sequences are in "alignment," i.e. when a minimal number of "null" bases have been inserted in the subject and/or reference sequences so as to maximize the number of existing bases in correspondence between the sequences. "Null" bases are not part of the subject and reference sequences; also, the minimal number of "null" bases inserted in the subject sequence may differ from the minimal number inserted in the reference sequence. In this definition, a reference sequence is considered "related" to a subject sequence where both amino acid sequences make up proteins or portions of proteins which are either αTAG antibodies or antibody fragments with αTAG binding affinity.

Each of the proteins comprising these aTAG antibodies or antibody fragments may independently be antibodies or antibody fragments or bi- or multi-functional proteins, e.g., such as fusion proteins, bi- and multi-specific antibodies, single chain antibodies, and the like.

Nucleic acids, amino acids, peptides, protective groups, active groups and so on, when abbreviated, are abbreviated according to the IUPAC IUB (Commission on Biological Nomenclature) or the practice in the fields concerned.

The basic immunoglobulin structural unit is set forth in Figure 1. The terms "constant" and "variable" are used functionally. The variable regions of both light ($V_L$) and heavy ($V_H$) chains determine binding recognition and specificity to the antigen. The constant region domains of light ($C_L$) and heavy ($C_H$) chains confer important biological properties such as antibody chain association, secretion, transplacental mobility, complement binding, binding to Fc receptors and the like.

The immunoglobulins of this invention have been developed to address the problems of the prior art. The methods of this invention produce, and the invention is directed to, composite antibodies. By "composite antibodies" is meant immunoglobulins comprising variable regions not hitherto found associated with each other in nature. By "composite Hum4 $V_L$, $V_H$ antibody" means an antibody or immunoreactive fragment thereof which is characterized by having at least a portion of the $V_L$ region encoded by DNA derived from the Hum4 $V_L$ germline gene and at least a portion of a $V_H$ region capable of combining with the $V_L$ to form a three dimensional structure having the ability to bind to TAG-72.

The composite Hum4 $V_L$, $V_H$ antibodies of the present invention assume a conformation having an antigen binding site which binds specifically and with sufficient strength to TAG-72 to form a complex capable of being isolated by using standard assay techniques (e.g., enzyme-linked immunosorbent

assay (ELISA), radioimmunoassay (RIA), or flourescence-activated cell sorter analysis (FACS), immunohistochemistry and the like). Preferably, the composite Hum4 $V_L$, $V_H$ antibodies of the present invention have an antigen binding affinity or avidity greater than $10^5$ $M^{-1}$, more preferably greater than $10^6$ $M^{-1}$ and most preferably greater than $10^8$ $M^{-1}$. For a discussion of the techniques for generating and reviewing immunoglobulin binding affinities see Munson (1983), Methods Enzymol., **92**:543-577 and Scatchard (1949), Ann. N.Y. Acad. Sci., **51**:660-672.

Human antibody kappa chains have been classified into four subgroups on the basis of invariant amino acid sequences (see, for example, Kabat *et al.* (1991), Sequences of Proteins of Immunological Interest (4th ed.), published by The U.S. Department of Health and Human Services). There appear to be approximately 80 human $V_K$ genes, but only one Subgroup IV $V_K$ gene has been identified in the human genome (see Klobeck, *et al.* (1985), Nucleic Acids Research, **13**:6516-6528). The nucleotide sequence of Hum4 $V_L$ is set forth in Kabat *et al.* (1991), *supra*.

It has been found, quite surprisingly, that an immunoglobulin having a light chain with at least a portion of the $V_L$ encoded by a gene derived from Hum4 $V_L$ may, if combined with a suitable $V_H$, have binding specificity for TAG-72.

The type of $J_L$ gene segment selected is not critical to the invention, in that it is expected that any $J_L$, if present, can associate with the Hum4 $V_L$. The present invention obviously contemplates the Hum4 $V_L$ in association with a human $J_K$ sequence. The five human $J_K$ sequences are set forth in Heiter *et al.* (1982), The Journal of Biological Chemistry, **357**:1516-1522. However, the present invention is not intended to be limited to the human $J_K$. The present invention specifically contemplates the Hum4 $V_L$ in association with any of the at

least six human $J_\lambda$ genes (see Hollis *et al.* (1982), Nature, **296**:321-325).

An exemplary technique for engineering the Hum4 $V_L$ with selected $J_L$ segments includes synthesizing a primer having a so-called "wagging tail", that does not hybridize with the target DNA; thereafter, the sequences are amplified and spliced together by overlap extension (see Horton *et al.* (1989), Gene, **77**:61-68).

The $C_L$ of the composite Hum4 $V_L$, $V_H$ antibodies is not critical to the invention. To date, the Hum4 $V_L$ has only been reported as having been naturally rearranged with the single $C_k$ gene (see Heiter *et al.* (1980), **Cell**, **22**:197-207). However, the present invention is not intended to be limited to the Ck light chain constant domain. That is, the $C_L$ gene segment may also be any of the at least six $C_\lambda$ genes (see Hollis *et al.*, *supra*).

The DNA encoding the heavy chain variable region consists roughly of a heavy chain variable ($V_H$) gene sequence, a heavy chain diversity ($D_H$) gene sequence, and a heavy chain joining ($J_H$) gene sequence.

The present invention is directed to any $V_H$ capable of combining with a light chain variable region effectively homologous to the light chain variable region encoded by the human Subgroup IV germline gene, to form a three dimensional structure having the ability to bind to TAG-72.

The choice of $D_H$ and $J_H$ segment of the composite Hum4 $V_L$, $V_H$ antibody are not critical to the present invention. Obviously, human and murine $D_H$ and $J_H$ gene segments are contemplated, provided that a given combination does not significantly decrease binding to TAG-72. Specifically, when utilizing CC46 $V_H$, CC49 $V_H$, CC83 $V_H$ and CC92 $V_H$, the composite Hum4 $V_L$, $V_H$ antibody will be designed to utilize the $D_H$ and $J_H$ segments which naturally associated with those $V_H$ of the respective hybridomas (see Figures 2 and 3). Exemplary murine

and human $D_H$ and $J_H$ sequences are set forth in Kabat *et al.* (1991), *supra*. An exemplary technique for engineering such selected $D_H$ and $J_H$ segments with a $V_H$ sequence of choice includes synthesizing selected oligonucleotides, annealing and ligating in a cloning procedure (see, Horton *et al.*, *supra*).

In a specific embodiment the composite Hum4 $V_L$, $V_H$ antibody will be a "composite Hum4 $V_L$, $V_H\alpha$TAG antibody", means an antibody or immunoreactive fragment thereof which is characterized by having at least a portion of the $V_L$ region encoded by DNA derived from the Hum4 $V_L$ germline gene and at least a portion of the $V_H$ region encoded by DNA derived from the $V_H\alpha$TAG germline gene, which is known in the art (see, for example, EPO 0 365 997 to Mezes *et al.*, the Dow Chemical Company). Figure 2 shows the nucleotide sequence of $V_H\alpha$TAG, and the nucleotide sequences encoding the $V_H$ of the CC46, CC49, CC83 and CC92 antibodies, respectively. Figure 3 shows the corresponding amino acid sequences of $V_H\alpha$TAG, CC46 $V_H$, CC49 $V_H$, CC83 $V_H$ and CC92 $V_H$.

A comparison of the nucleotide and amino acid sequences of $V_H\alpha$TAG, CC46 $V_H$, CC49 $V_H$, CC83 $V_H$ and CC92 $V_H$ shows that those CC antibodies are derived from $V_H\alpha$TAG. Somatic mutations occurring during productive rearrangement of the $V_H$ derived from $V_H\alpha$TAG in a B cell gave rise to some nucleotide changes that may or may not result in a homologous amino acid change between the productively rearranged hybridomas (see, EPO 0 365 997).

Because the nucleotide sequences of the $V_H\alpha$TAG and Hum4 $V_L$ germline genes have been provided herein, the present invention is intended to include other antibody genes which are productively rearranged from the $V_H\alpha$TAG germline gene. Other antibodies encoded by DNA derived from $V_H\alpha$TAG may be identified by using a hybridization probe made from the DNA or RNA of the $V_H\alpha$TAG or rearranged genes containing the recombined

$V_H\alpha$TAG. Specifically, the probe will include of all or a part of the $V_H\alpha$TAG germline gene and its flanking regions. By "flanking regions" is meant to include those DNA sequences from the 5' end of the $V_H\alpha$TAG to the 3' end of the upstream gene, and from 3' end of the $V_H\alpha$TAG to the 5' end of the downstream gene.

The CDR from the variable region of antibodies derived from $V_H\alpha$TAG may be grafted onto the FR of selected $V_H$, i.e., FR of a human antibody (see EPO 0 239 400 to Winter). For example, the cell line, B17X2, expresses an antibody utilizing a variable light chain encoded by a gene derived from Hum4 $V_L$ and a variable heavy chain which makes a stable $V_L$ and $V_H$ combination (see Marsh *et al.* (1985), Nucleic Acids Research, **13**:6531-6544; and Polke *et al.* (1982), Immunobiol. **163**:95-109. The nucleotide sequence of the $V_H$ chain for B17X2 is shown in Figure 4. The B17X2 cell line is publicly available from Dr. Christine Polke, Universitäts-Kinderklinik, Josef-Schneider-Str. 2, 8700 Würzburg, FRG). B17X2 is directed to N-Acetyl-D-Glucosamine and is not specific for TAG-72.

However, consensus sequences of antibody derived from the CDR1 of $V_H\alpha$TAG (amino acid residues 31 to 35 of Figure 3) may be inserted into B17X2 (amino acid residues 31 to 37 of Figure 4) and the CDR2 of $V_H\alpha$TAG (amino residues 50 to 65 of Figure 3) may be inserted into B17X2 (amino acid residues 52 to 67 of Figure 4). The CDR3 may be replaced by any $D_H$ and $J_H$ sequence which does not affect the binding of the antibody for TAG-72 but, specifically, may be replaced by the CDR3 of an antibody having its $V_H$ derived from $V_H\alpha$TAG, e.g., CC46, CC49, CC83 and CC92. Exemplary techniques for such replacement are set forth in Horton *et al.*, *supra*.

The $C_H$ domains of immunoglobulin heavy chain derived from $V_H\alpha$TAG genes, for example may be changed to a human sequence by known techniques (see, USP 4,816,567 to Cabilly, Genentech).

$C_H$ domains may be of various complete or shortened human isotypes, i.e., IgG (e.g., $IgG_1$, $IgG_2$, $IgG_3$, and $IgG_4$), IgA (e.g., IgA1 and IgA2), IgD, IgE, IgM, as well as the various allotypes of the individual groups (see Kabat *et al.* (1991), *supra*).

Given the teachings of the present invention, it should be apparent to the skilled artisan that human $V_H$ genes can be tested for their ability to produce an anti-TAG-72 immunoglobilin combination with the Hum4 $V_L$ gene. The $V_L$ may be used to isolate a gene encoding for a $V_H$ having the ability to bind to TAG-72 to test myriad combinations of Hum4 $V_L$ and $V_H$ that may not naturally occur in nature, e.g., by generating a combinatorial library using the Hum4 $V_L$ gene to select a suitable $V_H$. Examples of these enabling technologies include screening of combinatorial libraries of $V_L$-$V_H$ combinations using an Fab or single chain antibody (SCFV) format expressed on the surfaces of fd phage (Clackson, *et al.* (1991), Nature, **352**:624-628), or using a l phage system for expression of Fv's or Fabs (Huse, *et al.* (1989), Science, **246**:1275-1281). However, according to the teachings set forth herein, it is now possible to clone SCFV antibodies in *E. coli*, and express the SCFVs as secreted soluble proteins. SCFV proteins produced in *E. coli* that contain a Hum4 $V_L$ gene can be screened for binding to TAG-72 using, for example, a two-membrane filter screening system (Skerra, *et al.* (1991), Analytical Biochemistry, **196**:151-155).

The desired gene repertoire can be isolated from human genetic material obtained from any suitable source, e.g., peripheral blood lymphocytes, spleen cells and lymph nodes of a patient with tumor expressing TAG-72. In some cases, it is desirable to bias the repertoire for a preselected activity, such as by using as a source of nucleic acid, cells (source cells) from vertebrates in any one of various stages of age, health and immune response.

Cells coding for the desired sequence may be isolated, and genomic DNA fragmented by one or more restriction enzymes. Tissue (e.g., primary and secondary lymph organs, neoplastic tissue, white blood cells from peripheral blood and hybridomas) from an animal exposed to TAG-72 may be probed for selected antibody producing B cells. Variability among B cells derived from a common germline gene may result from somatic mutations occurring during productive rearrangement.

Generally, a probe made from the genomic DNA of a germline gene or rearranged gene can be used by those skilled in the art to find homologous sequences from unknown cells. For example, sequence information obtained from Hum4 $V_L$ and $V_H\alpha$TAG may be used to generate hybridization probes for naturally-occurring rearranged V regions, including the 5′ and 3′ nontranslated flanking regions. The genomic DNA may include naturally-occurring introns for portions thereof, provided that functional splice donor and splice acceptor regions had been present in the case of mammalian cell sources.

Additionally, the DNA may also be obtained from a cDNA library. mRNA coding for heavy or light chain variable domain may be isolated from a suitable source, either mature B cells or a hybridoma culture, employing standard techniques of RNA isolation. The DNA or amino acids also may be synthetically synthesized and constructed by standard techniques of annealing and ligating fragments (see Jones, *et al.* (1986), Nature, **321**:522-525; Reichmann *et al.*, (1988), Nature, **332**:323-327; Sambrook *et al.* (1989), *supra* and Merrifield *et al.* (1963), J. Amer. Chem. Soc., **85**:2149-2154). Heavy and light chains may be combined *in vitro* to gain antibody activity (see Edelman, *et al.* (1963), Proc. Natl. Acad. Sci. USA, **50**:753).

The present invention also contemplates a gene library of $V_H\alpha$TAG homologs, preferably human homologs of $V_H\alpha$TAG. By

"homolog" is meant a gene coding for a $V_H$ region (not necessarily derived from, or even effectively homologous to, the $V_H\alpha$TAG germline gene) capable of combining with a light chain variable region effectively homologous to the light chain variable region encoded by the human Subgroup IV germline gene, to form a three dimensional structure having the ability to bind to TAG-72.

Preferably, the gene library is produced by a primer extension reaction or combination of primer extension reactions as described herein. The $V_H\alpha$TAG homologs are preferably in an isolated form, that is, substantially free of materials such as, for example, primer extension reaction agents and/or substrates, genomic DNA segments, and the like. The present invention thus is directed to cloning the $V_H\alpha$TAG -coding DNA homologs from a repertoire comprised of polynucleotide coding strands, such as genomic material containing the gene expressing the variable region or the messenger RNA (mRNA) which represents a transcript of the variable region. Nucleic acids coding for $V_H\alpha$TAG -coding homologs can be derived from cells producing IgA, IgD, IgE, IgG or IgM, most preferably from IgM and IgG, producing cells.

The $V_H\alpha$TAG -coding DNA homologs may be produced by primer extension. The term "primer" as used herein refers to a polynucleotide whether purified from a nucleic acid restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complimentary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymer-ization such as DNA polymerase, reverse transcriptase and the like, and at a suitable temperature and pH.

Preferably, the $V_H\alpha$TAG -coding DNA homologs may be produced by polymerase chain reaction (PCR) amplifi-cation of double stranded genomic or cDNA, wherein two primers are used

for each coding strand of nucleic acid to be exponentially amplified. The first primer becomes part of the nonsense (minus or complementary) strand and hybridizes to a nucleotide sequence conserved among $V_H$ (plus) strands within the repertoire. PCR is described in Mullis *et al.* (1987), Meth. Enz., **155**:335-350; and PCR Technology, Erlich (ed.) (1989). PCR amplification of the mRNA from antibody-producing cells is set forth in Orlandi *et al.* (1989), Proc. Natl. Acad. Sci., USA, **86**:3387-3837.

According to a preferred method, the $V_H\alpha$TAG -coding DNA homologs are connected via linker to form a SCFV having a three dimensional structure capable of binding TAG-72. The SCFV construct can be in a $V_L$-L-$V_H$ or $V_H$-L-$V_L$ configuration. For a discussion of SCFV see Bird *et al.* (1988), Science, **242**:423-426. The design of suitable peptide linker regions is described in U.S. Patent No. 4,704,692 to Ladner *et al.*, Genex.

The nucleotide sequence of a primer is selected to hybridize with a plurality of immunoglobulin heavy chain genes at a site substantially adjacent to the $V_H\alpha$TAG -coding DNA homolog so that a nucleotide sequence coding for a functional (capable of binding) polypeptide is obtained. The choice of a primer's nucleotide sequence depends on factors such as the distance on the nucleic acid from the region coding for the desired receptor, its hybridization site on the nucleic acid relative to any second primer to be used, the number of genes in the repertoire it is to hybridize to, and the like. To hybridize to a plurality of different nucleic acid strands of $V_H\alpha$TAG -coding DNA homolog, the primer must be a substantial complement of a nucleotide sequence conserved among the different strands.

The peptide linker may be coded for by the nucleic acid sequences that are part of the poly-nucleotide primers used to prepare the various gene libraries. The nucleic acid sequence

coding for the peptide linker can be made up of nucleic acids attached to one of the primers or the nucleic acid sequence coding for the peptide linker may be derived from nucleic acid sequences that are attached to several polynucleotide primers used to create the gene libraries. Additionally, noncomplementary bases or longer sequences can be interspersed into the primer, provided the primer sequence has sufficient complemen-tarily with the sequence of the strand to be synthesized or amplified to non-randomly hybridize therewith and thereby form an extension product under polynucleotide synthesizing conditions (see Horton *et al.* (1989), Gene, **77**:61-68).

Exemplary human $V_H$ sequences from which complementary primers may be synthesized are set forth in Kabat *et al.* (1991), *supra*; Humphries *et al.* (1988), Nature, **331**:446-449; Schroeder *et al.* (1990), Proc. Natl. Acad. Sci. USA, **87**:6146-6150; Berman *et al.* (1988), EMBO Journal, **7**:727-738; Lee *et al.* (1987), J. Mol. Biol., **195**:761-768); Marks *et al.* (1991), Eur. J. Immunol., **21**:985-991; Willems, *et al.* (1991), J. Immunol., **146**:3646-3651; and Person *et al.* (1991), Proc Natl. Acad. Sci. USA, **88**:2432-2436. To produce $V_H$ coding DNA homologs, first primers are therefore chosen to hybridize to (i.e. be complementary to) conserved regions within the J region, CH1 region, hinge region, CH2 region, or CH3 region of immunoglobulin genes and the like. Second primers are therefore chosen to hydribidize with a conserved nucleotide sequence at the 5' end of the $V_H\alpha$TAG -coding DNA homolog such as in that area coding for the leader or first framework region.

Alternatively, the nucleic acid sequences coding for the peptide linker may be designed as part of a suitable vector. As used herein, the term "expression vector" refers to a nucleic acid molecule capable of directing the expression of genes to which they are operatively linked. The choice of vector to which a $V_H\alpha$TAG -coding DNA homologs is operatively

linked depends directly, as is well known in the art, on the functional properties desired, e.g., replication or protein expression, and the host cell (either procaryotic or eucaryotic) to be transformed, these being limitations inherent in the art of constructing recombinant DNA molecules. In preferred embodiments, the eucaryotic cell expression vectors used include a selection marker that is effective in an eucaryotic cell, preferably a drug resistant selection marker.

Expression vectors compatible with procaryotic cells are well known in the art and are available from several commercial sources. Typical of vector plasmids suitable for procaryotic cells are pUC8, pUC9, pBR322, and pBR329 available from BioRad Laboratories, (Richmond, CA), and pPL and pKK223 available from Pharmacia, (Piscataway, NJ).

Expression vectors compatible with eucaryotic cells, preferably those compatible with vertebrate cells, can also be used. Eucaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA homologue. Typical of vector plasmids suitable for eucaryotic cells are pSV2neo and pSV2gpt (ATCC), pSVL and pKSV-10 (Pharmacia), pBPV-1/PML2d (International Biotechnologies, Inc.), and pTDT1 (ATCC).

The use of viral expression vectors to express the genes of the $V_H\alpha$TAG -coding DNA homologs is also contemplated. As used herein, the term "viral expression vector" refers to a DNA molecule that includes a promoter sequences derived from the long terminal repeat (LTR) region of a viral genome. Exemplary phage include l phage and fd phage (see, Sambrook, *et al.* (1989), Molecular Cloning: A Laboratory Manual, (2nd ed.), and McCafferty *et al.* (1990), Nature, **6301**:552-554.

The population of $V_H\alpha TAG$ -coding DNA homologs and vectors are then cleaved with an endonuclease at shared restriction sites. A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instance, complementary cohesive termini can be engineered into the $V_H\alpha TAG$ -coding DNA homologs during the primer extension reaction by use of an appropriately designed polynucleotide synthesis primer, as previously discussed. The complementary cohesive termini of the vector and the DNA homolog are then operatively linked (ligated) to produce a unitary double stranded DNA molecule.

The restriction fragments of Hum4 $V_L$-coding DNA and the $V_H\alpha TAG$ -coding DNA homologs population are randomly ligated to the cleaved vector. A diverse, random population is produced with each vector having a $V_H\alpha TAG$ -coding DNA homolog and Hum4 $V_L$-coding DNA located in the same reading frame and under the control of the vector's promoter.

The resulting single chain construct is then introduced into an appropriate host to provide amplifi-cation and/or expression of a composite Hum4 $V_L$, $V_H\alpha TAG$ homolog single chain antibody. Transformation of appropriate cell hosts with a recombinant DNA molecule of the present invention is accomplished by methods that typically depend on the type of vector used. With regard to transformation of procaryotic host cells, see, for example, Cohen *et al.* (1972), Proceedings National Academy of Science, USA, 69:2110; and Sambrook, *et al.* (1989), *supra*. With regard to the transformation of vertebrate cells with retroviral vectors containing rDNAs, see for example, Sorge *et al.* (1984), Mol. Cell. Biol., **4**:1730-1737; Graham *et al.* (1973), Virol., **52**:456; and Wigler *et al.* (1979), Proceedings National Academy of Sciences, USA, **76**:1373-1376.

Exemplary prokaryotic strains that may be used as hosts include *E. coli*, *Bacilli*, and other entero-bacteriaceae such

as *Salmonella typhimurium*, and various *Pseudomonas*. Common eukaryotic microbes include *S. cerevisiae* and *Pichia pastoris*. Common higher eukaryotic host cells include Sp2/0, VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 and MDCK cell lines. Furthermore, it is now also evident that any cell line producing Hum4 $V_L$, e.g., the B17X2 human cell line, can be used as a recipient human cell line for introduction of a $V_H$ gene complementary to the Hum4 $V_L$ which allows binding to TAG-72. For example, the B17X2 heavy chain may be genetically modified to not produce the endogenous heavy chain by well known methods; in this way, glycosylation patterns of the antibody produced would be human and not non-human derived.

Successfully transformed cells, i.e., cells containing a gene encoding a composite Hum4 $V_L$, $V_H\alpha$TAG homolog single chain antibody operatively linked to a vector, can be identified by any suitable well known technique for detecting the binding of a receptor to a ligand. Preferred screening assays are those where the binding of the composite Hum4 $V_L$, $V_H\alpha$TAG homolog single chain antibody to TAG-72 produces a detectable signal, either directly or indirectly. Screening for productive Hum4 $V_L$ and $V_H\alpha$TAG homolog combinations, or in other words, testing for effective antigen binding sites to TAG-72 is possible by using for example, a radiolabeled or biotinylated screening agent, e.g., antigens, anti-bodies (e.g., B72.3, CC49, CC83, CC46, CC92, CC30, CC11 and CC15) or anti-idiotypic antibodies (see Huse *et al.*, *supra*, and Sambrook *et al.*, *supra*); or the use of marker peptides to the $NH_2$- or COOH-terminus of the SCFV construct (see Hopp *et al.* (1988), Biotechnology, **6**:1204-1210).

Of course, the Hum4 $V_L$-coding DNA and the $V_H\alpha$TAG -coding DNA homologs may be expressed as individual polypeptide chains (e.g., Fv) or with whole or fragmented constant regions (e.g., Fab, and $F(ab')_2$). Accordingly, the Hum4 $V_L$-coding DNA and the

$V_H\alpha TAG$ -coding DNA homologs may be individually inserted into a vector containing a $C_L$ or $C_H$ or fragment thereof, respectively. For a teaching of how to prepare suitable vectors see EPO 0 365 997 to Mezes *et al.*, The Dow Chemical Company.

DNA sequences encoding the light chain and heavy chain of the composite Hum4 $V_L$, $V_H$ antibody may be inserted into separate expression vehicles, or into the same expression vehicle. When coexpressed within the same organism, either on the same or the different vectors, a functionally active Fv is produced. When the $V_H\alpha TAG$ -coding DNA homolog and Hum4 $V_L$ polypeptides are expressed in different organisms, the respective polypeptides are isolated and then combined in an appropriate medium to form a Fv. See Greene *et al.*, Methods in Molecular Biology, Vol. 9, Wickner *et al.* (ed.); and Sambrook *et al.*, *supra*).

Subsequent recombinations can be effected through cleavage and removal of the Hum4 $V_L$-coding DNA sequence to use the $V_H\alpha TAG$ -coding DNA homologs to produce Hum4 $V_L$-coding DNA homologs. To produce a Hum4 $V_L$-coding DNA homolog, first primers are chosen to hybridize with (i.e. be complementary to) a conserved region within the J region or constant region of immunoglobulin light chain genes and the like. Second primers become part of the coding (plus) strand and hybridize to a nucleotide sequence conserved among minus strands. Hum4 $V_L$-coding DNA homologs are ligated into the vector containing the $V_H\alpha TAG$ -coding DNA homolog, thereby creating a second population of expression vectors. The present invention thus is directed to cloning the Hum4 $V_L$-coding DNA homologs from a repertoire comprised of polynucleotide coding strands, such as genomic material containing the gene expressing the variable region or the messenger RNA (mRNA) which represents a transcript of the variable region. It is thus possible to use an iterative process to define yet further, composite

antibodies, using later generation $V_H\alpha$TAG -coding DNA homologs and Hum4 $V_L$-coding DNA homologs.

The present invention further contemplates genetically modifying the antibody variable and constant regions to include effectively homologous variable region and constant region amino acid sequences. Generally, changes in the variable region will be made in order to improve or otherwise modify antigen binding properties of the receptor. Changes in the constant region of the antibody will, in general, be made in order to improve or otherwise modify biological properties, such as complement fixation, interaction with membranes, and other effector functions.

"Effectively homologous" refers to the concept that differences in the primary structure of the variable region may not alter the binding character-istics of the antibody. Normally, a DNA sequence is effectively homologous to a second DNA sequence if at least 70 percent, preferably at least 80 percent, and most preferably at least 90 percent of the active portions of the DNA sequence are homologous. Such changes are permissable in effectively homologous amino acid sequences so long as the resultant antibody retains its desired property.

If there is only a conservative difference between homologous positions of sequences, they can be regarded as equivalents under certain circumstances. General categories of potentially equivalent amino acids are set forth below, wherein amino acids within a group may be substituted for other amino acids in that group: (1) glutamic acid and aspartic acid; (2) hydrophobic amino acids such as alanine, valine, leucine and isoleucine; (3) asparagine and glutamine; (4) lysine and arginine and (5) threonine and serine.

Exemplary techniques for nucleotide replacement include the addition, deletion or substitution of various nucleotides, provided that the proper reading frame is maintained. Exemplary techniques include using polynucleotide-mediated, site-directed mutagenesis, i.e., using a single strand as a

template for extension of the oligonucleotide to produce a strand containing the mutation (see Zoller *et al.* (1982), Nuc. Acids Res., **10**:6487-6500; Norris *et al.* (1983), Nuc. Acids Res., **11**:5103-5112; Zoller *et al.* (1984), DNA, **3**:479-488; and Kramer *et al.* (1982), Nuc. Acids Res., **10**:6475-6485) and polymerase chain reaction exponentially amplifying DNA *in vitro* using sequence specified oligo-nucleotides to incorporate selected changes (see PCR Technology: Principles and Applications for DNA Amplification, Erlich, (ed.) (1989); and Horton *et al.*, *supra*).

Further, the antibodies may have their constant region domain modified, ie., the $C_L$, $CH_1$, hinge, $CH_2$, $CH_3$ and/or $CH_4$ domains of an antibody polypeptide chain may be deleted, inserted or changed (see EPO 327 378 A1 to Morrison *et al.*, the Trustees of Columbia University; USP 4,642,334 to Moore *et al.*, DNAX; and USP 4,704,692 to Ladner *et al.*, Genex).

Once a final construct is obtained, the composite Hum4 $V_L$, $V_H$ antibodies may be produced in large quantities by injecting the host cell into the peritoneal cavity of pristane-primed mice, and after an appropriate time (about 1-2 weeks), harvesting ascites fluid from the mice, which yields a very high titer of homogeneous composite Hum4 $V_L$, $V_H$ antibodies, and isolating the composite Hum4 $V_L$, $V_H$ antibodies by methods well known in the art (see Stramignoni *et al.* (1983), Intl. J. Cancer, **31**:543-552). The host cell are grown *in vivo*, as tumors in animals, the serum or ascites fluid of which can provide up to about 50 mg/mL of composite Hum4 $V_L$, $V_H$ antibodies. Usually, injection (preferably intraperitoneal) of about $10^6$ to $10^7$ histocompatible host cells into mice or rats will result in tumor formation after a few weeks. It is possible to obtain the composite Hum4 $V_L$, $V_H$ antibodies from a fermentation culture broth of procaryotic and eucaryotic cells, or from inclusion bodies of *E. coli* cells (see Buckholz and Gleeson (1991), BIO/TECHNOLOGY, **9**:1067-1072. The composite Hum4 $V_L$, $V_H$ antibodies can then be collected and

processed by well-known methods (see generally, Immunological Methods, vols. I & II, eds. Lefkovits, I. and Pernis, B., (1979 & 1981) Academic Press, New York, N.Y.; and Handbook of Experimental Immunology, ed. Weir, D., (1978) Blackwell Scientific Publications, St. Louis, MO).

The composite Hum4 $V_L$, $V_H$ antibodies can then be stored in various buffer solutions such as phosphate buffered saline (PBS), which gives a generally stable antibody solution for further use.

Uses

While it is possible for an antibody or fragment thereof to be administered alone –i.e. because they bear human $C_H$ regions and will thus exert effector functions including complement mediated cytotoxicity and antibody dependent cell-mediated cytotoxicity -- it is preferable to present it as a pharmaceutical formulation. The active ingredient may comprise, for topical administration, from 0.001% to 10% w/w, e.g., from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w but preferably not in excess of 5% w/w and more preferably from 0.1% to 1% w/w of the formulation.

The topical formulations of the present invention, comprise an active ingredient together with one or more acceptable carrier(s) therefor and optionally any other therapeutic ingredients(s). The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin to the site of where treatment is required, such as liniments, lotions, creams, ointments or pastes, and drops suitable for administration to the eye, ear, or nose.

Drops according to the present invention may comprise sterile aqueous or oily solutions or suspensions and may be prepared by dissolving the active ingredient in a suitable aqueous solution of a bactericidal and/or fungicidal agent and/or any other suitable preservative, and preferably including a surface active agent. The resulting solution may then be clarified and sterilized by filtration and transferred to the container by an aseptic technique. Examples of bactericidal and fungicidal agents suitable for inclusion in the drops are phenylmercuric nitrate or acetate (0.002%), benzalkonium chloride (0.01%) and chlorhexidine acetate (0.01%). Suitable solvents for the preparation of an oily solution include glycerol, diluted alcohol and propylene glycol.

Lotions according to the present invention include those suitable for application to the skin or eye. An eye lotion may comprise a sterile aqueous solution optionally containing a bactericide and may be prepared by methods similar to those for the preparation of drops. Lotions or liniments for application to the skin may also include an agent to hasten drying and to cool the skin, such as an alcohol or acetone, and/or a moisturizer such as glycerol or an oil such as castor oil or arachis oil.

Creams, ointments or pastes according to the present invention are semi-solid formulations of the active ingredient for external application. They may be made by mixing the active ingredient in finely-divided or powdered form, alone or in solution or suspension in an aqueous or non-aqueous fluid, with the aid of suitable machinery, with a greasy or non-greasy basis. The basis may comprise hydrocarbons such as hard, soft or liquid paraffin, glycerol, beeswax, a metallic soap; a mucilage; an oil of natural origin such as almond, corn, arachis, castor or olive oil; wool fat or its derivatives, or a fatty acid such as stearic or oleic acid together with an alcohol such as propylene glycol or

macrogels. The formulation may incorporate any suitable surface active agent such as an anionic, cationic or non-ionic surface active such as sorbitan esters or polyoxyethylene derivatives thereof. Suspending agents such as natural gums, cellulose derivatives or inorganic materials such as silicaceous silicas, and other ingredients such as lanolin, may also be included.

Kits according to the present invention include frozen or lyophilized humanized antibodies or humanized antibody fragments to be reconstituted, respectively, by thawing (optionally followed by further dilution) or by suspension in a (preferably buffered) liquid vehicle. The kits may also include buffer and/or excipient solutions (in liquid or frozen form) -- or buffer and/or excipient powder preparations to be reconstituted with water - for the purpose of mixing with the humanized antibodies or humanized antibody fragments to produce a formulation suitable for administration. Thus, preferably the kits containing the humanized antibodies or humanized antibody fragments are frozen, lyophilized, pre-diluted, or pre-mixed at such a concentration that the addition of a predetermined amount of heat, of water, or of a solution provided in the kit will result in a formulation of sufficient concentration and pH as to be effective for *in vivo* or *in vitro* use in the treatment or diagnosis of cancer. Preferably, such a kit will also comprise instructions for reconstituting and using the humanized antibody or humanized antibody fragment composition to treat or detect cancer. The kit may also comprise two or more component parts for the reconstituted active composition. For example, a second component part - in addition to the humanized antibodies or humanized antibody fragments - may be bifunctional chelant, bifunctional chelate, or a therapeutic agent such as a radionuclide, which when mixed with the humanized antibodies or humanized antibody fragments forms a conjugated system therewith. The above-noted buffers, excipients, and other

component parts can be sold separately or together with the kit.

It will be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of a humanized antibody or humanized antibody fragment of the invention will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the particular animal being treated, and that such optima can be determined by conventional techniques. It will also be appreciated by one of skill in the art that the optimal course of treatment, i.e., the number of doses of an antibody or fragment thereof of the invention given per day for a defined number of days, can be ascertained by those skilled in the art using conventional course of treatment determination tests.

The subject humanized antibodies may also be administered in combination with other anti-cancer agents, e.g., other antibodies or drugs. Also, the subject humanized antibodies or fragments may be directly or indirectly attached to effector moieties having therapeutic activity. Suitable effector moieties include by way of example cytokines (IL-2, TNF, interferons, colony stimulating factors, IL-1, etc.), cytotoxins (Pseudomonas exotoxin, ricin, abrin, etc.), radionuclides, such as $^{90}Y$, $^{131}I$, $^{99m}Tc$, $^{111}In$, $^{125}I$, among others, drugs (methotrexate, daunorubicin, doxorubicin, etc.), immunomodulators, therapeutic enzymes (e.g., beta-galactosidase), anti-proliferative agents, etc. The attachment of antibodies to desired effectors is well known. See, e.g., U.S. Patent No. 5,435,990 to Cheng et al. Moreover, bifunctional linkers for facilitating such attachment are well known and widely available. Also, chelators (chelants and chelates) providing for attachment of radionuclides are well known and available.

The composite Hum4 $V_L$, $V_H$ antibodies provide unique benefits for use in a variety of cancer treat-ments. In

addition to the ability to bind specifically to malignant cells and to localize tumors and not bind to normal cells such as fibroblasts, endothelial cells, or epithelial cells in the major organs, the composite Hum4 $V_L$, $V_H$ antibodies may be used to greatly minimize or eliminate HAMA responses thereto. Moreover, TAG-72 contains a variety of epitopes and thus it may be desirable to administer several different composite Hum4 $V_L$, $V_H$ antibodies which utilize a variety of VH in combination with Hum4 $V_L$.

Specifically, the composite Hum4 $V_L$, $V_H$ antibodies are useful for, but not limited to, *in vivo* and *in vitro* uses in diagnostics, therapy, imaging and biosensors.

The composite Hum4 $V_L$, $V_H$ antibodies may be incorporated into a pharmaceutically acceptable, non-toxic, sterile carrier. Injectable compositions of the present invention may be either in suspension or solution form. In solution form the complex (or when desired the separate components) is dissolved in a pharmaceutically acceptable carrier. Such carriers comprise a suitable solvent, preservatives such as benzyl alcohol, if needed, and buffers. Useful solvents include, for example, water, aqueous alcohols, glycols, and phosphonate or carbonate esters. Such aqueous solutions generally contain no more than 50 percent of the organic solvent by volume.

Injectable suspensions require a liquid suspending medium, with or without adjuvants, as a carrier. The suspending medium can be, for example, aqueous polyvinyl-pyrrolidone, inert oils such as vegetable oils or highly refined mineral oils, or aqueous carboxymethylcellulose. Suitable physio-logically-acceptable adjuvants, if necessary to keep the complex in suspension, may be chosen from among thickeners such as carboxymethylcellulose, polyvinyl-pyrrolidone, gelatin and the alginates. Many surfactants are also useful as suspending agents, for example, lecithin, alkylphenol, polyethylene oxide adducts,

naphthalenesulfonates, alkylbenzenesulfonates, and the polyoxyethylene sorbitan esters. Many substances which effect the hydrophobicity, density, and surface tension of the liquid suspension medium can assist in making injectable suspensions in individual cases. For example, silicone antifoams, sorbitol, and sugars are all useful suspending agents.

Methods of preparing and administering conjugates of the composite Hum4 $V_L$, $V_H$ antibody, and a therapeutic agent are well known or readily determined. Moreover, suitable dosages will depend on the age and weight of the patient and the therapeutic agent employed and are well known or readily determined.

Conjugates of a composite Hum4 $V_L$, $V_H$ antibody and an imaging marker may be administered in a pharma-ceutically effective amount for the *in vivo* diagnostic assays of human carcinomas, or metastases thereof, in a patient having a tumor that expresses TAG-72 and then detecting the presence of the imaging marker by appropriate detection means.

Administration and detection of the conjugates of the composite Hum4 $V_L$, $V_H$ antibody and an imaging marker, as well as methods of conjugating the composite Hum4 $V_L$, $V_H$ antibody to the imaging marker are accomplished by methods readily known or readily determined. The dosage of such conjugate will vary depending upon the age and weight of the patient. Generally, the dosage should be effective to visualize or detect tumor sites, distinct from normal tissues. Preferably, a one-time dosage will be between 0.1 mg to 200 mg of the conjugate of the composite Hum4 $V_L$ anti-body and imaging marker per patient.

Examples of imaging markers which can be conjugated to the composite Hum4 $V_L$ antibody are well known and include substances which can be detected by diagnostic imaging using a gamma scanner or hand held gamma probe, and substances which can be detected by nuclear magnetic resonance imaging using a nuclear magnetic resonance spectrometer.

Suitable, but not limiting, examples of substances which can be detected using a gamma scanner include $^{125}I$, $^{131}I$, $^{123}I$, $^{111}In$, $^{105}Rh$, $^{153}Sm$, $^{67}Cu$, $^{67}Ga$, $^{166}Ho$, $^{177}Lu$, $^{186}Re$, $^{188}Re$ and $^{99m}Tc$. An example of a substance which can be detected using a nuclear magnetic resonance spectrometer is gadolinium.

Conjugates of a composite Hum4 $V_L$, $V_H$ anti-bodies and a therapeutic agent may be administered in a pharmaceutically effective amount for the *in vivo* treatment of human carcinomas, or metastases thereof, in a patient having a tumor that expresses TAG-72. A "pharmaceutically effective amount" of the composite Hum4 $V_L$ antibody means the amount of said antibody (whether unconjugated, i.e., a naked antibody, or conjugated to a therapeutic agent) in the pharmaceutical composition should be sufficient to achieve effective binding to TAG-72.

Exemplary naked antibody therapy includes, for example, administering heterobifunctional composite Hum4 $V_L$, $V_H$ antibodies coupled or combined with another antibody so that the complex binds both to the carcinoma and effector cells, e.g., killer cells such as T cells, or monocytes. In this method, the composite Hum4 $V_L$ antibody-therapeutic agent conjugate can be delivered to the carcinoma site thereby directly exposing the carcinoma tissue to the therapeutic agent. Alter-natively, naked antibody therapy is possible in which antibody dependent cellular cytoxicity or complement dependent cytotoxicity is mediated by the composite Hum4 $V_L$ antibody.

Examples of the antibody-therapeutic agent conjugates which can be used in therapy include antibodies coupled to radionuclides, such as $^{131}I$, $^{90}Y$, $^{105}Rh$, $^{47}Sc$, $^{67}Cu$, $^{212}Bi$, $^{211}At$, $^{67}Ga$, $^{125}I$, $^{186}Re$, $^{188}Re$, $^{177}Lu$, $^{99m}Tc$, $^{153}Sm$, $^{123}I$ and $^{111}In$; to drugs, such as methotrexate, adriamycin; to biological response modifiers, such as interferon and to toxins, such as ricin.

Methods of preparing and administering conjugates of the composite Hum4 $V_L$, $V_H$ antibodies and a therapeutic agent are well known or readily determined. The pharmaceutical composition may be administered in a single dosage or multiple dosage form. Moreover, suitable dosages will depend on the age and weight of the patient and the therapeutic agent employed and are well known or readily determined.

Composite Hum4 $V_L$, $V_H$ antibodies, and parti-cularly composite Hum4 $V_L$, $V_H$ single chain antibodies thereof, are particularly suitable for radioimmunoguided surgery (RIGS). In RIGS, an antibody labeled with an imaging marker is injected into a patient having a tumor that expresses TAG-72. The antibody localizes to the tumor and is detected by a hand-held gamma detecting probe (GDP). The tumor is then excised (see Martin *et al.* (1988), Amer. J. Surg., **156**:386-392; and Martin *et al.* (1986), Hybridoma, **5**:S97-S108). An exemplary GDP is the Neoprobe™ scanner, commercially available from Neoprobe Corporation, Columbus, OH. The relatively small size and human character of the composite Hum4 $V_L$, $V_H$ single chain antibodies will accelerate whole body clearance and thus reduce the waiting period after injection before surgery can be effectively initiated.

Administration and detection of the composite Hum4 $V_L$, $V_H$ antibody-imaging marker conjugate may be accomplished by methods well known or readily determined.

The dosage will vary depending upon the age and weight of the patient, but generally a one time dosage of 0.1 mg to 200 mg of the composite Hum4 $V_L$ antibody-marker conjugate per patient is administered.

## EXAMPLES

The following nonlimiting examples are merely for illustration of the construction and expression of composite Hum4 $V_L$, $V_H$ antibodies. All temperatures not otherwise indicated are Centigrade. All percents not otherwise indicated are by weight.

### Example 1

CC49 and CC83 were isolated from their respective hybridomas using pNP9 as a probe (see Figure 5). CC49 $V_H$ was obtained from p49 g1-2.3 (see Figure 6) and CC83 $V_H$ was obtained from p83 g1-2.3 (see Figure 7), following the procedures set forth in EPO 0 365 997.

DNA encoding an antibody light chain was isolated from a sample of blood from a human following the protocol of Madisen *et. al.* (1987), Am. J. Med. Genet., **27**:379-390), with several modifications. Two 5 mL purple-cap Vacutainer tubes (containing EDTA as an anticoagulant) were filled with blood and stored at ambient temperature for 2 hours. The samples were transferred to two 4.5 mL centrifuge tubes. To each tube was added 22.5 mL of filter-sterilized erythrocycte lysate buffer (0.155 M $NH_4Cl$ and 0.17 M Tris, pH 7.65, in a volume ratio of 9:1), and incubated at 37°C for 6.5 minutes The tubes became dark red due to the lysed red blood cells. The samples were centrifuged at 9°C for 10 minutes, using an SS-34 rotor and a Sorvall centrifuge at 5,300 revolutions per minute (rpm) (~3,400 x g). The resulting white cell pellets were resuspended in 25 mL of 0.15 M NaCl solution. The white blood cells were then centrifuged as before. The pellets were resuspended in 500 µL of 0.15 M NaCl and transferred to 1.5 mL microcentrifuge tubes. The cells were pelleted again for 3 minutes, this time in the microcentrifuge at 3,000 rpm. Very few red blood cells remained on the pellet. After the

supernatants were decanted from the 2 microcentrifuge tubes, 0.6 mL high TE buffer (100 mM Tris, pH 8.0) was added. The tubes were hand-shaken for between 10 and 15 minutes. The resulting viscous solution was extracted with phenol, phenol-chloroform and finally with just chloroform as described in Sambrook *et al., supra*. To 3.9 mL of pooled extracted DNA solution were added 0.4 mL NaOAc (3 M, pH 5), and 10 mL 100 percent ethanol. A white stringy precipitate was recovered with a yellow pipette tip, transferred into a new Eppendorf tube, washed once with 70 percent ethanol, and finally washed with 100 percent ethanol. The DNA was dried *in vacuo* for 1 minute and dissolved in 0.75 mL deionized water. A 20 µL aliquot was diluted to 1.0 mL and the OD 260 nm value was measured and recorded. The concentration of DNA in the original solution was calculated to be 0.30 mg/mL.

Oligonucleotides (oligos) were synthesized using phosphoramidite chemistry on a 380A DNA synthesizer (Applied Biosystems, Foster, CA) starting on 0.2 µM solid support columns. Protecting groups on the final products were removed by heating in concentrated ammonia solution at 55°C for 12 hours. Crude mixtures of oligonucleotides (approximately 12 OD 260 nm units) were applied to 16 percent polyacrylamide-urea gels and electrophoresed. DNA in the gels was visualized by short wave UV light. Bands were cut out and the DNA eluted by heating the gel pieces to 65°C for 2 hours. Final purification was achieved by application of the eluted DNA solution onto C-18 Sep-Pac™ columns (Millipore) and elution of the bound oligonucleotide with a 60 percent methanol solution. The pure DNA was dissolved in deionized, distilled water ($ddH_2O$) and quantitated by measuring OD 260 nm.

A GeneAmp™ DNA amplification kit (Cetus Corp., Emeryville, CA) was used to clone the Hum4 $V_L$ germline gene by the polymerase chain reaction (PCR), which was set up according to the manufacturer's directions. A thermal cycler was used for the denaturation (94 °C), annealing (45°C) and

elongation (72°C) steps. Each of the three steps in a cycle was carried out for 4 minutes; there was a total of 30 cycles.

Upstream of the regulatory sequences in the Hum4 $V_L$ germline gene, there is a unique *Cla* I restriction enzyme site. Therefore, the 5' end oligonucleotide for the PCR, called HUMVL(+) (Figure 8), was designed to include this *Cla* I site.

Figure 9 shows the human J4 (HJ4) amino acid and DNA sequences. The first two amino acids (Leu-Thr) complete the CDR3 region, the remainder make up the FR4 region. Glu is underlined in HJ4 because in CC49 J5 a somatic mutation had occured in this codon converting GAG (for Glu) to GTG (for Val). The (↓) indicates the slice site and the beginning of the intron between the J and Cκ exons. DNA sequences underlined in HJ4 represent parts of the sequence used for the 3' end PCR oligo.

Figure 10 is the DNA and amino acid sequence of Hum4 $V_L$ in human/chimeric CC49H and CC83H. Specifically, the figure shows the entire DNA sequence of the Hum4 $V_L$ gene *Cla* I-*Hind* III segment in pRL1001, clone #2. A single base difference occured at position 3461 and is marked by an asterik (*). The corresponding amino acid sequences in the coding exons are shown. The site of the Leu Pro mutation in clone #7 is boxed. An arrow (↑) indicates the site of the single base deletion in clone #11. The coding strand is underlined to designate the sites used for hybridization of complementary oligonucleotide primers. In order the primers occur from the 5' end as follows: HUMLIN1(-); HUMLIN2(-); HUMLCDR1(-) and *Hind* III Cκ(-) (not shown).

The 3' end oligonucleotide, called HUMVL(-) (Figure 8), contained a unique *Hind* III site; sufficient mouse intron sequence past the splicing site to permit an effective splice donor function; a human J4 sequence contiguous with the 3' end of the $V_L$ exon of Hum4 $V_L$ to complete the CDR3 and FR4

sequences of the $V_L$ domain (see Figures 9 and 10); nucleotides to encode a tyrosine residue at position 94 in CDR3; and 29 nucleotides close to the 3' end of the $V_L$ exon of Hum4 $V_L$ (shown underlined in the oligonucleotide HUMVL(-) in Figure 8) to anneal with the human DNA target. In total, this 3' end oligonucleotide for the PCR was 98 bases long with a non-annealing segment (a "wagging tail") of 69 nucleotides. A schematic of the Hum4 $V_L$ gene target and the oligonucleotides used for the PCR are shown in Figure 11. A 5'end oligo ($HUMV_L(+)$) and the 3'-end oligo ($HUMV_L(-)$) used to prime the elongation reactions for Taq polymerase and the target Hum4 $V_L$ gene are shown.

A PCR reaction was set up with 1 µg of total human DNA in a reaction volume of 100 µL. Primers HUMVL(-) and HUMVL(+) were each present at an initial concentration of 100 pmol. Prior to the addition of *Taq* polymerase (2.5 units/reaction) 100 µLs of mineral oil were used to overlay the samples. Control samples were set up as outlined below. The samples were heated to 95°C for 3 minutes. When the PCR was complete, 20 µL samples were removed for analysis by agarose gel electrophoresis.

Based on the known size of the Hum4 $V_L$ DNA fragment to be cloned, and the size of the oligonucleotides used to target the gene, a product of 1099 bp was expected. A band corresponding to this size was obtained in the reaction (shown in lane 7, Figure 12).

To prepare a plasmid suitable for cloning and subsequently expressing the Hum4 $V_L$ gene, the plasmid pSV2neo was obtained from ATCC and subsequently modified. pSV2neo was modified as set forth below (see Figure 13).

The preparation of pSV2neo-101 was as follows. Ten micrograms of purified pSV2neo were digested with 40 units of *Hind* III at 37°C for 1 hour. The linearized plasmid DNA was precipitated with ethanol, washed, dried and dissolved in 10 µL of water. Two microliters each of 10 mM dATP, dCTP, dGTP

and dTTP were added, as well as 2 µL of 10X ligase buffer (Stratagene, La Jolla, CA). Five units (1 µL) of DNA polymerase I were added to make blunt the *Hind* III sticky ends. The reaction mixture was incubated at room temperature for 30 minutes. The enzyme was inactivated by heating the mixture to 65°C for 15 minutes. The reaction mixture was then phenol extracted and ethanol precipitated into a pellet. The pellet was dissolved in 20 µL deionized, distilled water. A 2 µL aliquot (ca. 1 µg) was then added to a standard 20 µL ligation reaction, and incubated overnight at 4°C.

Competent *E. coli* DH1 cells (Invitrogen) were transformed with 1 µL and 10 µL aliquots of a ligation mix (Invitrogen, San Diego, CA) according to the manufacturer's directions. Ampicillin resistant colonies were obtained on LB plates containing 100 µg/mL ampicillin. Selected clones grown in 2.0 mL overnight cultures were prepared, samples of plasmid DNA were digested with *Hind* III and *Bam* HI separately, and a correct representative clone selected.

The resulting plasmid pSV2neo-101 was verified by size mapping and the lack of digestion with *Hind* III.

A sample of DNA (10 µg) from pSV2neo-101 mini-lysate was prepared by digesting with 50 units of *Bam* HI at 37 °C for 2 hours. The linearized plasmid was purified from a 4 percent polyacrylamide gel by electroelution. The DNA ends were made blunt by filling in the *Bam* HI site using dNTPs and Klenow fragment, as described earlier for the *Hind* III site of pSV2 neo-101.

A polylinker segment containing multiple cloning sites was incorporated at the *Bam* HI site of pSV2neo-101 to create pSV2neo-102, as shown in Figure 14. The arrow (←) indicates the direction of the *Eco* RI site in the vector. Note that the polylinker could be inserted in both orientations such that the *Bam* HI site on the left side could also be regenerated. The nucleotides used to fill-in the *Bam* HI site are shown in

italics. The top synthetic oligo was called (CH(+) while the complimentary strand was CH(-). Equimolar amounts of two oligonucleotides, CH(+) and CH(-) (shown in Figure 14) were annealed by heating for 3 minutes at 90°C and cooling to50 °C. Annealed linker DNA and blunt ended pSV2neo-101 were added, in a 40:1 molar volume, to a standard 20 µL ligation reaction. *E. coli* DH1 (Invitrogen) was transformed with 0.5 µL and 5 µL aliquots of the ligation mixture (Invitrogen). Twelve ampicillin resistant colonies were selected for analysis of plasmid DNA to determine whether the linker had been incorporated.

A *Hind* III digest of mini-lysate plasmid DNA revealed linker incorporation in six of the clones. The plasmid DNA from several clones was sequenced, to determine the number of linker units that were blunt-end ligated to pSV2neo-101 as well as the relative orientation(s) with the linker. Clones for sequencing were selected on the basis of positive digestion with *Hind* III.

A Sequenase™ sequencing kit (United States Biochemical Corp, Cleveland, OH) was used to sequence the DNA. A primer, NEO102SEQ, was used for sequencing and is shown in Figure 15. It is complementary to a sequence located upstream from the *Bam*HI site in the vector. The *Bam* HI site where the polylinker was inserted in pSV2neo-101 is boxed. Between 3 µg and 5 µg of plasmid DNA isolated from *E. coli* mini-lysates were used for sequencing. The DNA was denatured and precipitated prior to annealing, as according to the manufacturer's instructions. Electrophoresis was carried out at 1500 volts; gels were dried prior to exposure to Kodak X-ray film. Data was processed using a DNASIS™ computer program (Hitachi).

From the DNA sequence data of 4 clones analyzed (see photograph of autoradiogram representing the sequence data of 2 of these clones- Figure 16, reading the sequence (going up) is in the 5' to 3' direction of the (+) strand), compared to

the expected sequence in Figure 14, two clones having the desired orientation were obtained. In both cases a single 30-base linker unit was incorporated , but in opposite orientations. The panel A-sequence resulted in pSV2neo-120; and the panel B sequence resulted in pSV2neo-102. A representative clone was selected and designated pSV2neo-102.

A human Cκ gene was inserted into pSV2neo-102 to form pRL1000. The human Cκ DNA was contained in a 5.0 kb *Hind* III-*Bam* HI fragment (see Hieter *et al.* (1980), Cell, **22**:197-207).

A 3 µg sample of DNA from a mini-lysate of pSV2neo-102 was digested with *Bam* HI and *Hind* III. The vector DNA was separated from the small *Bam* HI-*Hind* III linker fragment, generated in the reaction, by electro-phoresis on a 3.75 percent DNA polyacrylamide gel. The desired DNA fragment was recovered by electroelution. A pBR322 clone containing the 5.0 kb *Hind* III-*Bam* HI fragment of the human Cκ gene (see Hieter *et al.*, *supra*) was designated phumCκ. The 5.0 kb *Hind* III-*Bam* HI fragment was ligated with pSV2neo-102 and introduced into *E. coli* DH1 (Invitrogen). Ampicillin resistant colonies were screened and a clone containing the human Cκ gene was designated pRL1000.

Finally, pRL1000 clones were screened by testing mini-lysate plasmid DNA from *E. coli* with *Hind* III and *Bam* HI. A clone producing a plasmid which gave 2 bands, one at 5.8 kb (representing the vector) and the other at 5.0 kb (representing the human Cκ insert) was selected. Further characterization of pRL1000 was achieved by sequencing downstream from the *Hind* III site in the intron region of the human Cκ insert. The oligonucleotide used to prime the sequencing reaction was NEO102SEQ (see Figure 15). Two hundred and seventeen bases were determined (see Figure 17). A new oligonucleotide corresponding to the (-) strand near the *Hind* III site (shown in Figure 17) was synthesized so that clones, containing the Hum4 $V_L$ gene that were cloned into the

*Cla* I and *Hind* III sites in pRL1000 (see Figure 13), could be sequenced.

A *Cla* I-*Hind* III DNA fragment containing Hum4 $V_L$ obtained by PCR was cloned into the plasmid vector pRL1000. DNA of pRL1000 and the Hum4 $V_L$ were treated with *Cla* I and *Hind* III and the fragments were gel purified by electrophoresis, as described earlier.

The pRL1000 DNA fragment and fragment containing Hum4 $V_L$ gene were ligated, and the ligation mixture used to transform *E. coli* DH1 (Invitrogen), following the manufacturer's protocol. Ampicillin resistant clones were screened for the presence of the Hum4 $V_L$ gene by restriction enzyme analysis and a representative clone designated pRL1001 (shown in Figure 18). This is the expression vector to introduce the human anti-tumor L chain gene in Sp2/0 cells.

Four plasmids having the correct *Cla* I-*Hind* III restriction pattern were analyzed further by DNA sequencing of the insert region (see Figure 19). *Hind* III CK(-) (shown by underlining on the plus strand to which it hybridizes in Figure 17), HUMLIN1(-) (shown by underlining on the plus strand to which it hybridizes in Figure 10), HUMLIN2(-) (shown by underlining on the plus strand to which it hybridizes in Figure 10) and HUMLCDR1(-) (shown by underlining on the plus strand to which it hybridizesin Figure 10) were used as the sequencing primers. Two out of the four plasmids analyzed had the expected sequence in the coding regions (Figure 19, clones 2 and 9). The gel is read in the 5' to 3' direction on the (-) strand, from the Hind III CK (-) primer. Clones 2 and 9 were equivalent to the expected sequence, clone 7 had a single base base substitution (marked by *) and clone 11 had a single base deletion (marked by →).

Clone 2 was chosen and used for generating sufficient plasmid DNA for cell transformations and other analysis. This plasmid was used for sequencing through the Hum4 $V_L$, and the

upstream region to the *Cla* I site. Only one change at nucleotide position 83 from a C to a G (Figure 10) was observed, compared to a published sequence (Klobeck *et al.* (1985), *supra*). The DNA sequence data also indicates that the oligonucleotides used for PCR had been correctly incorporated into the target sequence.

A Biorad Gene Pulser™ apparatus was used to transfect Sp2/0 cells with linearized plasmid DNAs containing the light or heavy chain constructs. The Hum4 $V_L$ was introduced into Sp2/O cells along with corresponding heavy chains by the co-transfection scheme indicated in Table 1.

Table 1

| Cell Line Designation | DNA Added | | |
|---|---|---|---|
| | L Chain | H Chain pRL1001 | H Chain p49 |
| MP1-44H | 20 µg | 15 µg | 0 µg |
| MP1-84H | 20 µg | 0 µg | 15 ug |

A total of 8.0 X $10^6$ Sp2/0 cells were washed in sterile PBS buffer (0.8 mL at 1 X $10^7$ viable cells/mL) and held on ice for 10 minutes. DNA of pRL1001, linearized at the *Cla* I site, and DNA of either p49 g1-2.3 or p83 g1-2.3, linearized at their respective *Nde* I sites, were added, in sterile PBS, to the cells (see protocol - Table 2) and held at 0 °C for a further 10 minutes. A single 200 volt, 960 µF electrical pulse lasting between 20 and 30 milliseconds was used for the electroporation. After holding the perturbed cells on ice for 5 minutes, 25 mL of RPMI medium with 10 percent fetal calf serum were introduced, and 1.0 mL samples aliquoted in a 24 well tissue culture plate. The cells were incubated at 37 °C in a 5 percent $CO_2$ atmosphere. After 48 hours, the media was exchanged with fresh selection media, now containing both 1 mg/mL Geneticin (G418) (Difco) and 0.3 µg/ml mycophenolic

acid/gpt medium. Resistant cells were cultured for between 7 and 10 days.

Supernatants from wells having drug resistant colonies were tested on ELISA plates for activity against TAG-72. A roughly 10 percent pure TAG-72 solution prepared from LSI74T tumor xenograft cells was diluted 1:40 and used to coat flexible polyvinyl chloride microtitration plates (Dynatech Laboratories, Inc.). Wells were air-dried overnight, and blocked the next day with 1 percent BSA. Supernatant samples to be tested for anti-TAG-72 antibody were added to the washed wells and incubated for between 1 and 2 hours at 37 °C. Alkaline phosphatase labeled goat anti-human IgG (diluted 1:250) (Southern Biotech Associates, Birmingham, AL) was used as the probe antibody. Incubation was for 1 hour. The substrate used was p-nitrophenylphosphate. Color development was terminated by the addition of 1.0 N NaOH. The plates were read spectrophotometrically at 405 nm and 450 nm, and the values obtained were 405 nm-450 nm.

Those samples producing high values in the assay were subcloned from the original 24 well plate onto 96 well plates. Plating was done at a cell density of half a cell per well (nominally 50 cells) to get pure monoclonal cell lines. Antibody producing cell lines were frozen down in media containing 10 percent DMSO.

Two cell lines were procured having the designations: MP1-44H and MP1-84H. MP1-44H has the chimeric CC49 g1 heavy chain with the Hum4 $V_L$ light chain; and MP1-84H has the chimeric CC83 g1 heavy chain with the Hum4 $V_L$ light chain.

A 1.0 L spinner culture of the cell line of the cell line MP1--44H was grown at 37 °C for 5 days for antibody production. The culture supernatant was obtained free of cells by centrifugation and filtration through a 0.22 micron filter apparatus. The clarified supernatant was passed over a Protein A cartridge (Nygene, New York). Immunoglobulin was eluted using 0.1 M sodium citrate buffer, pH 3.0. The pH of

the eluting fractions containing the antibody was raised to neutrality by the addition of Tris base, pH 9.0. The antibody-containing fractions were concentrated and passed over a Pharmacia Superose 12 HR 10/30 gel filtration column. A protein was judged to be homogeneous by SDS polyacrylamide gel electrophoresis. Isoelectric focusing further demonstrated the purity of MP1-44H.

The biological performance of the human composite antibody, MP1-44H, was evaluated by comparing immunohistochemistry results with two other anti-TAG-72 antibodies CC49 (ATCC No. HB 9459) and Ch44 (ATCC No. HB 9884). Sections of human colorectal tumor embedded in paraffin were tested with the three antibodies by methods familiar to those skilled in this art. All three antibodies gave roughly equivalent binding recognition of the tumor antigen present on the tumor tissue sample.

A further test of the affinity and biological integrity of the human composite antibody MP1-44H was a competition assay, based on cross-competing radioiodine-labeled versions of the antibody with CC49 and Ch44 in all combinations. From the data shown in Figure 20, it is apparent that the affinity of all 3 antibodies is equivalent and can bind effectively to tumor antigen.

MP1-44H (ATCC HB 10426) and MP1-84H (ATCC HB 10427) were deposited at the American Type Culture Collection (ATCC). The contract with ATCC provides for permanent availability of the cell lines to the public on the issuance of the U.S. patent describing and identifying the deposit or the publications or upon the laying open to the public of any U.S. or foreign patent application, whichever comes first, and for availability of the cell line to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 CFR §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of the present application has

agreed that if the cell lines on deposit should die or be lost or destroyed when cultivated under suitable conditions for a period of thirty (30) years or five (5) years after the last request, it will be promptly replaced on notification with viable replacement cell lines.

Example 2

Single-chain antibodies consist of a $V_L$, $V_H$ and a peptide linker joining the $V_L$ and $V_H$ domains to produce SCFVs. A single chain antibody, SCFV1, was constructed to have the Hum4 $V_L$ as V Domain 1 and CC49 $V_H$ as V Domain 2 (see Figure 21).

The polypeptide linker which joins the two V domains was encoded by DNA introduced at the 3' end of the Hum4 $V_L$ DNA during a PCR. The oligonucleotides SCFV1a and SCFV2 were designed to obtain the DNA segment incorporating part of the yeast invertase leader sequence, the Hum4 $V_L$ and the SCFV linker.

The polypeptide linker for SCFV1 was encoded in oligonucleotide SCFV1b (see below). The underlined portions of the oligonucleotides SCFV1a and SCFV1b are complementary to sequences in the Hum4 $V_L$ and linker respectively. The sequences of SCFV1a and SCFV1b are as follows, with the hybridizing sequences underlined:

SCFV1a with the *Hind* III in bold:

5'-CTGC**AAGCTT**CCTTTTCCTTTTGGCTGGTTTTG
CAGCCAAAATATCTGCAGACATCGTGATGACCCAGTC-3'

SCFV1b with the *Aat* II site in bold:

5'-CGTAA**GACGTC**TAAGGAACGAAATTGGGCCAATTGTTCTGAGGA
GACCGAACCTGACTCCTTCACCTTGGTCCCTCCGCCG-3'

The target DNA in the PCR was pRL1001 (shown in Figure 18). The PCR was performed pursuant to the teachings of Mullis *et al.*, *supra*. A DNA fragment containing the Hum4 $V_L$-linker DNA component for the construction of SCFV1 was obtained and purified by polyacrylamide gel electrophoresis according to the teachings of Sambrook *et al.*, *supra*.

p49 g1-2.3, containing CC49 $V_H$, was the target DNA in the PCR. PCR was performed according to the methods of Mullis *et al.*, *supra*. The oligonucleotides used for the PCR of CC49 $V_H$ are as follows, with the hybridizing sequences underlined:

SCFV1c, with the *Aat* II site in bold:

5'-CTTA**GACGTC**CAGTTGCAGCAGTCTGACGC-3'

SCFV1d, with the *Hind* III site in bold:

5'-GATC**AAGCTT**CACTAGGAGACGGTGACTGAGGTTCC-3'

The purified Hum4 $V_L$-linker and $V_H$ DNA fragments were treated with *Aat* II (New England Biolabs, Beverly, MA) according to the manufacturer's protocol, and purified from a 5 percent polyacrylamide gel after electrophoresis. An equimolar mixture of the *Aat* II fragments was ligated overnight. The T4 DNA ligase was heat inactivated by heating the ligation reaction mixture at 65 °C for 10 minutes. Sodium chloride was added to the mixture to give a final concentration of 50 mM and the mixture was further treated with *Hind* III. A *Hind* III DNA fragment was isolated and purified from a 4.5 percent polyacrylamide gel and cloned into a yeast expression vector (see Carter *et al.* (1987), In: DNA Cloning, A Practical Approach, Glover (ed.) Vol III: 141-161). The sequence of the fragment, containing the contiguous SCFV1 construct, is set forth in Figure 22.

The anti-TAG-72 SCFV1 described herein utilized the yeast invertase leader sequence (shown as positions -19 to -1 of Figure 22), the Hum4 $V_L$ (shown as positions 1 to 113 of Figure 22), an 18 amino acid linker (shown as positions 114 to 132 of

Figure 22) and CC49 $V_H$ (shown as positions 133 to 248 of Figure 22).

The complete DNA and amino acid sequence of SCFV1 is given in Figure 22. The oligonucleotides used to sequence the SCFV1 are set forth below.

TPI:

5'-CAATTTTTTGTTTGTATTCTTTTC-3'.

HUVKF3:

5'-CCTGACCGATTCAGTGGCAG-3'.

DC113:

5'-TCCAATCCATTCCAGGCCCTGTTCAGG-3'.

SUC2T:

5'-CTTGAACAAAGTGATAAGTC-3'.

Example 3

A plasmid, pCGS517 (Figure 23), containing a prorennin gene was digested with *Hind* III and a 6.5 kb fragment was isolated. The plasmid pCGS517 has a triosephosphate isomerase promoter, invertase [SUG2] signal sequence, the prorennin gene and a [SUC2] terminator. The *Hind* III-digested SCFV1 insert obtained above (see Figure 23) was ligated overnight with the *Hind* III fragment of pCGS517 using T4 DNA ligase (Stratagene, La Jolla, CA).

The correct orientation existed when the *Hind* III site of the insert containing part of the invertase signal sequence ligated to the vector DNA to form a gene with a contiguous signal sequence. *E. coli* DHI (Invitrogen) cells were transformed and colonies screened using a filter-microwave technique (see Buluwela, *et al.* (1989), Nucleic Acids Research, **17**:452). From a transformation plate having several hundred colonies, 3 positive clones were obtained. Digesting the candidate plasmids with *Sal* I and *Kpn* I, each a single cutter, differentiated between orientations by the size of the DNA fragments produced. A single clone, pDYSCFV1 (Figure 23), had the correct orientation and was used for further experimentation and cloning. The probe used was derived from

pRL1001, which had been digested with *Kpn* I and *Cla* I (see Figure 18). The probe DNA was labeled with $^{32}P$ a-dCTP using a random oligonucleotide primer labeling kit (Pharmacia LKB Biotechnology, Piscataway, NJ).

The next step was to introduce the *Bgl* II-*Sal* 1 fragment from pDYSCFV1 into the same restriction sites of another vector (ca. 9 kb), which was derived from PCGS515 (Figure 23), to give an autonomously replicating plasmid in *S. cerevisiae*.

DNA from the vector and insert were digested in separate reactions with *Bgl* II and *Sal* I using 10X buffer number 3 (50 MM Tris-HCI (pH 8.0), 100 mM NaCl, BRL). The DNA fragment from pDYSCFV1 was run in and electroeluted from a 5 percent polyacrylamide gel and the insert DNA was run and electroeluted from a 3.75 percent polyacrylamide gel. A standard ligation using T4 DNA ligase (Stratagene, La Jolla, CA) and a transformation using *E. coli* DH1 (Invitrogen) was carried out. Out of 6 clones selected for screening with *Bgl* II and *Sal* I, all 6 were correctly oriented, and one was designated pCGS515/SCFV1 (Figure 23).

DNA sequencing of pCGS515/SCFVI DNA was done using a Sequenase™ kit (U.S. Biochemical, Cleveland, OH) using pCGS515/SCFV1 DNA. The results have been presented in Figure 22 and confirm the sequence expected, based on the linker, the Hum4 $V_L$ and the CC49 $V_H$.

Transformation of yeast cells using the autonomosly replicating plasmid pCGS515/SCFV1 was carried out using the lithium acetate procedures described in Ito *et al.* (1983), J. Bacteriol., **153**:163-168; and Treco (1987), In: Curent Protocols in Molecular Biology, Ausebel *et al.* (eds), **2**:13.71-13.7.6. The recipient strain of *S. cerevisiae* was CGY1284 having the genotype - MAT a (mating strain a), ura 3-52 (uracil auxotrophy), SSC1-1 (supersecreting 1), and PEP4$^+$ (peptidase 4 positive).

Transformed clones of CGY1284 carrying SCFV plasmids were selected by their ability to grow on minimal media in the absence of uracil. Transformed colonies appeared within 3 to 5 days. The colonies were transferred, grown and plated in YEPD medium. Shake flasks were used to provide culture supernatant with expressed product.

An ELISA procedure was used to detect biological activity of the SCFV1. The assay was set up such that the SCFV would compete with biotinylated CC49 (biotin-CC49) for binding to the TAG-72 antigen on the ELISA plate.

SCFV1 protein was partially purified from a crude yeast culture supernatant, using a Superose 12 gel filtration column (Pharmacia LKB Biotechnology), and found to compete with biotinylated CC49 in the competition ELISA. These results demonstrate that the SCFV1 had TAG-72 binding activity.

The SCFV1 protein was detected by a standard Western protocol (see Towbin *et al.* (1979), Proc. Natl. Acad. Sci., USA, **76**:4350-4354). The detecting agent was biotinylated FAID14 (ATCC No. CRL 10256), an anti-idiotypic monoclonal antibody prepared from mice that had been immunized with CC49. A band was visualized that had an apparent molecular weight of approximately 26,000 daltons, the expected size of SCFV1. This result demonstrated that the SCFV1 had been secreted and properly processed.

Example 4

The following example demonstrates the cloning of human $V_H$ genes into a SCFV plasmid construct containing sequence coding for the Hum4 $V_L$ and a 25 amino acid linker called UNIHOPE.

A vector was prepared from plasmid pRW 83 containing a chloramphenicol resistance ($Cam^r$) gene for clone selection, and a *penP* gene with a *penP* promoter and terminator (see Mezes, *et al.* (1983), J. Biol. Chem., **258**:11211-11218) and the *pel B* signal sequence (see Lei, *et al.* (1987), *supra*). The vector

was designated Fragment A (see Figure 24). The *penP* gene was removed with a *Hind* III/*Sal* I digest.

The *penP* promoter and *pel B* signal sequence were obtained by a PCR using pRW 83 as a template and oligonucleotides penP1 and penP2 as primers. The fragment was designated Fragment B (see Figure 24). A *Nco* I enzyme restriction site was introduced at the 3' end of the signal sequence region by the penP2 oligonucleotide.

penP1:

5'-CGATAAGCTTGAATTCCATCACTTCC-3'

penP2:

5'-GGCCATGGCTGGTTGGGCAGCGAGTAATAACAATCCAGCG GCT
GCCGTAGGCAATAGGTATTTCATCAAAATCGTCTCCCTCCGTTTGAA-3'

A SCFV comprised of a Hum4 $V_L$, a CC49 $V_H$, and an 18 amino acid linker (Lys Glu Ser Gly Ser Val Ser Ser Glu Gln Leu Ala Gln Phe Arg Ser Leu Asp) was obtained from pCGS515/SCFV1 by PCR using oligonucleotides penP3 and penP6. This fragment was designated Fragment D (see Figure 24). A *Bcl* I site was introduced at the 3' end of the $V_H$ region by the penP6 oligonucleotide.

penP3:

5'-GCTGCCCAACCAGCCATGGCCGACATCGTGATGACCCAGTCTCC-3'

penP6(-):

5'-CTCTTGATCACCAAGTGACTTTATGTAAGATGATGTTTTG ACG
GATTCATCGCAATGTTTTTATTTGCCGGAGACGGTGACTGAGGTTCC-3'

Fragments B and D were joined by PCR using oligonucleotides penP1 and penP6, following the procedures of Horton *et al., supra*. The new fragment was designated E (See Figure 24).

Fragment C containing the *penP* termination codon was isolated by digesting pRW 83 with *Bcl* I and *Sal* I, and designated Fragment C. pRW 83 was isolated from *E. coli* strain GM161, which is DNA methylase minus or dam$^-$.

Plasmid pSCFV 31 (see Figure 24) was created with a three part ligation Fragments A, C, and E.

The *Nco* I restriction enzyme site within the Cam$^r$ gene and the *Hind* III site located at the 5' end of the *penP* promoter in pSCFV 31 were destroyed through a PCR DNA amplification using oligonucleotides Nco1.1 and Nco1.3(-) to generate an *Eco* RI-*Nco* I fragment and oligonucleotides Nco1.2 and Nco1.4c(-) to generate a *Nco* I to *Eco* RI fragment. These two fragments were joined by PCR-SOE using oligonucleotides Nco1.1 and Nco1.4c(-). The oligonucleotides are set forth below:

Nco1.1:

5'-TCCGGAATTCCGTATGGCAATGA-3'

Nco1.3(-):

5'-CTTGCGTATAATATTTGCCCATCGTGAAAACGGGGGC-3'

Nco1.2:

5'-ATGGGCAAATATTATACGCAAG-3'

Nco1.4c(-):

5'-CACTGAATTCATCGATGATAAGCTGTCAAACATGAG-3'

pSCFV 31 was digested with *Eco* RI and the larger fragment was isolated by polyacrylamide gel electrophoresis. To prevent self ligation, the DNA was dephosphorylated using calf intertinal alkaline phosphatase according to the teachings of Sambrook *et al., supra*.

A two part ligation of the larger pSCFV 31 digested fragment and the PCR-SOE fragment, described above, resulted in the creation of pSCFV 31b (see Figure 25).

pSCFV 31b was digested with *Nco* I and *Sal* I and a fragment containing the Cam$^r$ gene was isolated.

The Hum4 $V_L$ was obtained by PCR DNA amplification using pCGS515/SCFV1 as a template and oligonucleotides 104BH1 and 104BH2(-) as primers.

104BH1:

5'-CAGCCATGGCCGACATCGTGATGACCCAGTCTCCA-3'

104BH2(-):

5'-AAGCTTGCCCCATGCTGCTTTAACGTTAGTTTTATCTGCTGG
AGACAGAGTGCCTTCTGCCTCCACCTTGGTCCCTCCGCCGAAAG-3'

The CC49 $V_H$ was obtained by PCR using p49 g1-2.3 (Figure 5) as a template and oligonucleotides 104B3 and 104B4(-) as primers. A *Nhe* I enzyme restriction site was introduced just past the termination codon in the 3' end (before the *Bcl* I site) by oligonucleotide 104B4(-).

104B3:

5'-GTTAAAGCAGCATGGGGCAAGCTTATGACTCAGTTGCAGCAGTCTGACGC-3'

104B4(-):

5'-CTCTTGATCACCAAGTGACTTTATGTAAGATGATGTTTTGACGGATT
CATCGCTAGCTTTTTATTTGCCATAATAAGGGGAGACGGTGACTGAGGTTCC-3'

In the PCR which joined these two fragments using oligonucleotides 104BH1 and 104B4(-) as primers, a coding region for a 22 amino acid linker was formed.

A fragment C (same as above) containing the *penP* termination codon was isolated from pRW 83 digested with *Bcl* I and *Sal* I.

Plasmid pSCFV 33H (see figure 25) was created with a three part ligation of the vector, fragment C, and the SCFV fragment described above.

pSCFV 33H was digested with *Nco*I and *Nhe*I, and the DNA fragment containing the Cam$^r$ gene was isolated as a vector.

Hum4 $V_L$ was obtained by PCR DNA amplification using pRL1001 (see Figure 18) as a template and oligonucleotides UNIH1 and UNIH2(-) as primers. Oligonucleotides for the PCR were:

UNIH1:

5'-CAG**CCATGG**CCGACATTGTGATGTCACAGTCTCC-3'

The *Nco* I site is in bold and the hybridizing sequence is underlined.

UNIH2(-):

5'-GAGGTCCGTAAGATCTGCCTCGCTACCTAGCAAA
AGGTCCTC**AAGCTT**GATCACCACCTTGGTCCCTCCGC-3'

The *Hind* III site is in bold.

The CC49 $V_H$ was obtained by a PCR using p49 g1-2.3 (see Figure 6) as a template and oligonucleotides UNI3 and UNI4(-) as primers.

UNI3:

5'-AGCGAGGCAGATCTTACGGAC**CTCGAG**GTTCAGTTGCAGCAGTCTGAC-3'.

The *Xho* I site is in bold and the hybridizing sequence is underlined.

UNI4(-):

5'-CATC**GCTAGC**TTTTTATGAGGAGACGGTGACTGAGGTTCC-3'.

The *Nhe* I site is in bold and the hybridizing sequence is underlined.

Oligonucleotides UNIH1 and UNI4(-) were used in the PCR-SOE amplification which joined the Hum4 $V_L$ and CC49 $V_H$ fragments and formed a coding region for a negatively charged fifteen amino acid linker. The DNA was digested with *Nhe* I and *Nco* I and ligated with the vector fragment from the *Nco* I-

*Nhe* I digest of pSCFV 33H. The resultant plasmid was designated pSCFV UNIH (shown in Figure 25).

With the construction of pSCFV UNIH, a universal vector for any SCFV was created with all the desired restriction enzyme sites in place.

pSCFV UNIH was digested with *Hind* III/*Xho* I, and the large DNA fragment containing the $Cam^r$ gene, Hum4 $V_L$ and CC49 $V_H$ was isolated.

A fragment coding for a 25 amino acid linker, was made by annealing the two oligonucleotides shown below. The linker UNIHOPE is based on 205C SCA™ linker (see Whitlow, (1990) Antibody Engineering: New Technology and Application Implications, IBC USA Conferences Inc, MA), but the first amino acid was changed from serine to leucine and the twenty-fifth amino acid were was changed from glycine to leucine, to accomodate the *Hind* III and *Xho* I restriction sites. The nucleotide sequence of the single chain portion of pSCFV Unihope H is shown in Figure 26. Structural sequences are indicated by the amino acid sequence written above the DNA sequence. The symbols _ and _ indicate the beginning and end of a given segment. The amino acid sequence of the linker is boxed.

The nucleotide sequence encoding the linker UNIHOPE is set forth below:

UNIHOPE (Figure 26):

5'-TATAAAGCTTAGTGCGGACGATGCGAAAAAGGATGCTGCGAAG
AAGGATGACGCTAAGAAAGACGATGCTAAAAAGGACCTCGAGTCTA-3'

UNIHOPE(-) (Figure 26):

5'-TAGACTCGAGGTCCTTTTTAGCATCGTCTTTCTTAGCGTCAT
CCTTCTTCGCAGCATCCTTTTTCGCATCGTCCGCACTAAGCTTTATA-3'

The resulting strand was digested with *Hind* III/*Xho* I and ligated into the vector, thus generating the plasmid pSCFV UHH (shown in Figure 27). Plasmid pSCFV UHH expresses a

biologically active, TAG-72 binding SCFV consisting of the Hum1 $V_L$ and CC49 $V_H$. The expression plasmid utilizes the ß-lactamase *penP* promoter, pectate lyase *pelB* signal sequence and the *penP* terminator region. Different immunoglobulin light chain variable regions can be inserted in the *Nco* I-*Hind* III restriction sites, different SCFV linkers can be inserted in the *Hind* III-*Xho* I sites and different immunoglobulin heavy chain variable regions can be inserted in the *Xho* I-*Nhe* I sites.

*E. coli* AG1 (Stratagene) was transformed with the ligation mix, and after screening, a single chloramphenicol resistant clone, having DNA with the correct restriction map, was used for further work.

The DNA sequence and deduced amino acid sequence of the SCFV gene in the resulting plasmid are shown in Figure 26.

*E. coli* AG1 containing pSCFV UHH were grown in 2 ml of LB broth with 20 µg/mL chloramphenicol (CAM 20). The culture was sonicated and assayed using a competition ELISA. The cells were found to produce anti-TAG-72 binding material. The competition assay was set up as follows: a 96 well plate was derivatized with a TAG-72 preparation from LS174T cells. The plate was blocked with 1% BSA in PBS for 1 hour at 31 °C and then washed 3 times. Twenty-five microliters of biotin CC49 (1/20,000 dilution of a 1 mg/mL solution) were added to the wells along with 25 µL of sample to be tested (competition step) and the plate was incubated for 30 minutes at 31 °C. The relative amounts of TAG-72 bound to the plate, biotinylated CC49, streptavidin-alkaline phosphatase, and color development times were determined empirically in order not to have excess of either antigen or biotinylated CC49, yet have enough signal to detect competition by SCFV. Positive controls were CC49 at 5 µg/mL and CC49 Fab at 10 µL/mL. Negative controls were 1% BSA in PBS and/or concentrated LB. At the end of the competition step, unbound proteins were washed away.

Fifty microliters of a 1:1000 dilution of streptavidin conjugated with alkaline phosphatase (Southern Biotechnology Associates, Inc., Birmingham, AL) were added and the plate was incubated for 30 minutes at 31 °C. The plate was washed 3 more times. Fifty microliters of a para-nitrophenylphosphate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were added and the color reaction was allowed to develop for a minimum of 20 minutes. The relative amount of SCFV binding was measured by optical density scanning at 405-450 nm using a microplate reader (Molecular Devices Corporation, Menlo Park, CA). Binding of the SCFV resulted in decreased binding of the biotinylated CC49 with a concomitant decrease in color development. The average value for triplicate test samples is shown in the table below:

| Sample (50 µL) (mixed 1:1 with CC49 Biotin) | OD 405 nm - OD 450 nm Value at 50 minutes |
|---|---|
| Sonicate *E. coli* AG1/ pSCFVUHH clone 10 | 0.072 |
| Sonicate *E. coli* AG1/ pSCFVUHH clone 11 | 0.085 |
| CC49 at 5 mg/mL | 0.076 |
| CC49 Fab at 10 mg/mL | 0.078 |
| LB (negative control) | 0.359 |

The data indicates that there was anti-TAG-72 activity present in the *E. coli* AGI/pSCFVUHH clone sonicate.

Example 5

The plasmid pSCFVUHH may be used to host other $V_H$ genes on *Xho* I-*Nhe* I fragments and test in a SCFV format, following the procedures set forth below. A schematic for this process is shown in Figure 31.

Isolating total RNA from peripheral blood lymphocytes:

Blood from a normal, healthy donor is drawn into three 5 mL purple-cap Vacutainer tubes. Seven mL of blood are added to two 15 mL polypropylene tubes. An equal volume of lymphoprep (cat# AN5501, Accurate) is added and the solution is mixed by inversion. Both tubes are centrifuged at 1000 rpm and 18 °C for 20 minutes. The resulting white area near the top of the liquid (area not containing red blood cells) is removed from each sample and placed into two sterile polypropylene centrifuge tube. Ten mL of sterile PBS are added and the tube mixed by inversion. The samples are centrifuged at 1500 rpm and 18 °C for 20 minutes Total RNA is isolated from resulting pellet according to the RNAzol B Method (Chomczynski and Sacchi (1987), Analytical Biochemistry, **162**:156-159). Briefly, the cell pellets are lysed in 0.4 mL RNAzol solution (cat#:CS-105, Cinna/Biotecx). RNA is solubilized by passing the cell pellet through a 1 mL pipet tip. Sixty µL of chloroform are added and the solution is shaken for 15 seconds. RNA solutions are then placed on ice for 5 minutes. Phases are separated by centrifugation at 12000 x g and 4 °C for 15 minutes. The upper (aqueous) phases are transferred to fresh RNase-free microcentrifuge tubes. One volume of isopropanol is added and the samples placed at -20 °C for 1 hour. The samples are then placed on dry ice for 5 minutes and finally centrifuged for 40 seconds at 14,000 x g and 4 °C. The resulting supernatant is removed from each sample and the pellet is dissolved in 144 µL of sterile RNase-

free water. Final molarity is brought to 0.2 in NaCl. The DNA is reprecipitated by adding 2 volumes of 100% ethanol, leaving on dry ice for 10 minutes, and centrifugation at 14,000 rpm and 4 °C for 15 minutes. The supernatants are then removed, the pellets washed with 75% ethanol and centrifuged for 8 minutes at 12000 x g and 4 °C. The ethanol is then removed and the pellets dried under vacuum. The resulting RNA is then dissolved in 20 sterile water containing 1 µl RNasin (cat#:N2511, Promega).

cDNA synthesis:

cDNA synthesis is performed using a Gene Amp™ PCR kit (cat#: N808-0017 Perkin Elmer Cetus), RNasin™ (cat#: N2511, Promega), and AMV reverse transcriptase (cat#: M9004, Promega). The following protocol is used for each sample:

| Components | Amount |
|---|---|
| $MgCl_2$ solution | 4 µl |
| 10 X PCR buffer II | 2 µl |
| dATP | 2 µl |
| dCTP | 2 µl |
| dGTP | 2 µl |
| dTTP | 2 µl |
| 3' primer (random hexamers) | 1 µl |
| RNA sample | 2 µl |
| RNasin | 1 µl |
| AMV RT | 1.5 µl |

Samples are heated at 80 °C for 3 minutes then slowly cooled to 48 °C. The samples are then centrifuged for 10 seconds. AMV reverse transcriptase is added to the samples which are then incubated for 30 minutes at 37 °C. After

incubation, 0.5 µl of each dNTP and 0.75 reverse transcriptase (cat#:109118, Boehringer Mannheim) are added. The samples are incubated for an additional 15 minutes at 37 °C.

PCR Reaction:

Oligonucleotides are designed to amplify human $V_H$ genes by polymerase chain reaction. The 5' oligonucleotides are set forth below:

HVH 135:

5'-TATTCTCGAGGTGCA(AG)CTG(CG)TG(CG)AGTCTGG-3'

HVH2A:

5'-TATTCTCGAGGTCAA(CG)TT(AG)A(AG)GGAGTCTGG-3'

HVH46:

5'-TATTCTCGAGGTACAGCT(AG)CAG(CG)(AT)GTC(ACG)GG-3'

The 3' oligonucleotides are set forth below:

JH1245:

5'-TTATGCTAGCTGAGGAGAC(AG)GTGACCAGGG-3'

JH3:

5'-TTATGCTAGCTGAAGAGACGGTGACCATTG

JH6:

5'-TTATGCTAGCTGAGGAGACGGTGACCGTGG-3'

PCR reactions are performed with a GeneAmp™ PCR kit (cat#:N808-0017, Perkin Elmer Cetus). Components are listed below:

| Components | Amount |
|---|---|
| $ddH_2O$ | 75 µl |
| 10 x buffer | 10 µl |
| dATP | 2 µl |
| dCTP | 2 µl |
| dGTP | 2 µl |
| dTTP | 2 µl |
| 1* Target DNA | 1 µl |
| 2* 5′ primer | 2.5 µl |
| 3′ primer | 2.0 µl |
| 3* AmpliTaq™ Polymerase | 1.3 µl |

| SUBSTANCE | AMOUNT |
|---|---|
| DNA | 20 µl |
| NEB Buffer #2 | 4.5 µl |
| *Nhe* I | 2 µl |
| *Xho* I | 2 µl |
| $ddH_2O$ | 16.5 µl |

Samples are incubated at 37 °C for one hour. After this incubation, an additional 1.5 µL *Nhe* I is added and samples are incubated an additional two hours at 37 °C.

Purification of DNA:

After the restriction enzyme digest, DNA is run on a 5 percent polyacrylamide gel (Sambrook *et al.* (1989), *supra*). Bands of 390-420 bp in size are excised from the gel. DNA is electroeluted and ethanol precipitated according to standard procedures.

PCR products resulting from oligonucleotide combinations are pooled together: JH1245 with HVH135, HVH2A and HVH46; JH3 with HVH135, HVH2A and HVH46; JH6 with HVH135, HVH2A and HVH46. The volume of the resulting pools are reduced under vacuum to 50 microliters. The pools are then purified from a

4 percent polyacrylamide gel (Sambrook *et al.* (1989), *supra*) to isolate DNA fragments. Bands resulting at 390-420 bp are excised from the gel. The DNA from excised gel slices is electroeluted according to standard protocols set forth in Sambrook, *supra*.

Isolation of pSCFVUHH *Xho* I/*Nhe* I Vector Fragment

Approximately 5 µg in 15 µL of pSCFVUHH plasmid is isolated using the Magic Mini-prep™ system (Promega). To this is added 5.4 µL OF 10X Buffer #2 (New England Biolabs), 45 units of *Xho* I (New England Biolabs), 15 units of *Nhe* I and 24 µL of $ddH_2O$. The reaction is allowed to proceed for 1 hour at 37 °C. The sample is loaded on a 4% polyacrylamide gel, electrophoresed and purified by electroelution, as described earlier. The DNA pellet is dissolved in 20 µL of $ddH_2O$.

One hundred nanograms of pSCFVUHH digested with *Xho* I/*Nhe* I is ligated with a 1:1 molar ratio of purified human $V_H$ inserts digested with *Xho* I and *Nhe* I using T4 DNA ligase (Stratagene). Aliquots are used to transform competent *E. coli* AG1 cells (Stratagene) according to the supplier's instructions.

GVWP hydrophilic membranes (cat# GVWP14250, Millipore) are placed on CAM 20 LB agar plates (Sambrook *et al.*, 1989). One membrane is added to each plate. Four hundred microliters of the *E. coli* AG1 transformation suspension from above are evenly spread over the surface of each membrane. The plates are incubated for 16 hours at 37 °C.

Preparation of TAG-72-coated membranes:

A 1% dilution of partially purified tumor associated glycoprotein-72 (TAG-72) produced in LS174 T-cells is prepared in TBS (cat# 28376, Pierce). Ten milliliters of the TAG dilution are placed in a petri plate (cat# 8-757-14, Fisher) for future use. Immobilon-P PVDF transfer membranes (cat#

SE151103, Millipore) are immersed in methanol. The membranes are then rinsed three times in sterile double distilled water. After the final wash, the excess water is allowed to drain. Each of the membranes are placed in 10 milliliters of dilute TAG-72. The membranes are incubated at ambient temperature from 1 hour with gentle shaking. After incubation, the membranes are blocked with Western blocking solution (25 mM Tris, 0.15 M NaCl, pH 7.6; 1% BSA) for about 1 hour at ambient temperature.

Blocking solution is drained from the TAG membranes. With the side exposed to TAG-72 facing up, the membranes are placed onto fresh CAM 20 plates. Resulting air pockets are removed. The bacterial membranes are then added, colony side up, to a TAG membrane. The agar plates are incubated for 24 to 96 hours at ambient temperatures.

The orientation of the TAG-72 and bacterial membranes are marked with permanent ink. Both membranes are removed from the agar surface. The TAG-72 membrane is placed in 20 ml of Western antibody buffer (TBS in 0.05% Tween-20, cat# P-1379, Sigma Chemical Co.; 1% BSA, cat#3203, Biocell Laboratories) containing 0.2 ng of CC49-Biotin probe antibody. The bacterial membranes are replaced on the agar surface in their original orientation and set aside. CC49-Biotin is allowed to bind to the TAG membranes for 1 hour at 31 °C with gentle shaking. The membranes are then washed three times with TTBS (TBS, 0.05% Tween-20) for 5 minutes on an orbital shaker at 300 rpm. Streptavidin alkaline phosphatase (cat# 7100-04, Southern Biotechnology Associates) is added to Western antibody buffer to produce a 0.1% solution. The TAG-72 membranes are each immersed in 16 milliliters of the streptavidin solution and allowed to incubate for 30 minutes at 31 °C with gentle shaking. After incubation, the membranes are washed as previously described. A final wash is then performed using Western alkaline phosphate buffer (8.4 g $NaCO_3$, 0.203 g $MgCl_2$-$H_2O$, pH 9.8), for 2 minutes at 200 rpm at ambient

temperature. To develop the membranes, Western blue stabilized substrate (cat# S384B, Promega) is added to each membrane surface. After 30 minutes at ambient temperatures, development of the membranes is stopped by rinsing the membranes three times with $ddH_2O$. The membranes are then photographed and clear zones are corelated with colonies on the hydrophilic membrane, set aside earlier. Colony(ies) are isolated for growth in culture and used to prepare plasmid DNA for sequencing characterization. Also, the protein product is isolated to evaluate specificity and affinity.

Identification of Hum4 $V_L$, human $V_H$ combinations using pATDFLAG.

In a second assay system, Hum4 $V_L$ - human $V_H$ combinations are discovered that bind to TAG-72 according to the schematic, *supra*, except for the following a different plasmid vector, pATDFLAG, was used (see below): at the assay step, IBI MII antibody is used as a probe to detect any Hum4 $V_L$ - $V_H$ SCFV combinations that have bound to the hydrophobic membrane coated with TAG-72 and a sheep anti-mouse Ig antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) is used to detect any binding of the MII antibody to TAG-72.

The plasmid pATDFLAG was generated from pSCFVUHH (see Figure 29) to incorporate a Flag-coding sequence 3' of any human $V_H$ genes to be expressed contiguously with Hum4 $V_L$. The plasmid pATDFLAG, when digested with *Xho* I and *Nhe* I and purified becomes the human $V_H$ discovery plasmid containing Hum4 $V_L$ in this SCFV format. The plasmid pATDFLAG was generated as follows. Plasmid pSCFVUHH treated with *Xho* I and *Nhe* I (isolated and described above) was used in a ligation reaction with the annealed FLAG and FLAGNC oligonucleotides.

FLAGC:

5'-TCGAGACAATGTCGCTAGCGACTACAAGGACGATGATGACAAATAAAAAC-3'

FLAGNC:

5'-CTAGGTTTTTATTTGTCATCATCGTCCTTGTAGTCGCTAGCGACATTGTC-3'

Equimolar amounts (1 x $10^{-10}$ moles of each of the oligonucleotides FLAGC and FLAGNC were mixed together using a ligation buffer (Stratagene). The sample is heated to 94 °C and is allowed to cool to below 35 °C before use in the ligation reaction below.

Ligation Reaction to Obtain pATDFLAG

| COMPONENT | AMOUNT |
|---|---|
| pSCFVUHH *Xho* I/*Nhe* I vector | 1.5 µl |
| ANNEALED FLAGC/FLAGNC | 0.85 µl |
| 10X Ligation buffer | 2 µl |
| T4 DNA LIGASE | 1 µl |
| 10 MM ATP | 2 µl |
| ddH$_2$O | 12.65 µl |

This ligation reaction is carried out using the following components and amounts according the ligation protocol disclosed above. *E. coli* AG1 cells (Stratagene) are transformed with 3 µl of the above ligation reaction and colonies selected using CAM 20 plates. Clones having appropriate *Nhe* I, *Xho* I and *Nhe* I/*Xho* I restriction patterns are selected for DNA sequencing.

The oligonucleotide used to verify the sequence of the FLAG linker in pATDFLAG (see Figure 28) is called PENPTSEQ: 5'-CTTTATGTAAGATGATGTTTTG-3. This oligonucleotide is derived from the non-coding strand of the *pen*P terminator region. DNA sequencing is performed using Sequenase™ sequencing kit (U.S. Biochemical, Cleveland, OH) following the manufacturer's directions. The DNA and deduced amino acid sequences of the Hum4 $V_L$ - UNIHOPE linker - FLAG peptide of pATDFLAG is shown in Figure 28.

Generation of pSC49FLAG

The CC49$V_H$ is inserted into the sites of *Xho* I - *Nhe* I pATDFLAG (see Figure 29) and evaluated for biological activity with the purpose of serving as a positive control for the FLAG assay system to detect binding to TAG-72. The new plasmid, called pSC49FLAG (see Figure 29) is generated as follows. The plasmid pATDFLAG (5 mg, purified from a 2.5 ml culture by the Magic Miniprep™ system (Promega) is treated with *Xho* I and *Nhe* I and the large vector fragment purified as described above for pSCFVUHH. The CC49 $V_H$ insert DNA fragment is obtained by PCR amplification from pSCFVUHH and oligonucleotides UNI3 as the 5' end oligonucleotide and SC49FLAG as the 3' end oligonucleotide. The resulting DNA and amino acid sequences of this SCFV antibody, with the FLAG peptide at the C-terminus, is shown in Figure 30. The PCR reaction is carried out using 100 pmol each of the oligonucleotides, 0.1 ng of pSCFVUHH target DNA (uncut) and the standard protocol and reagents provided by Perkin Elmer Cetus. The DNA is first gel

purfied, then treated with *Xho* I and *Nhe* I to generate sticky ends and purified from a 4% polyacrylamide gel and electroeluted as described earlier. The DNA vector (pATDFLAG treated with *Xho* I and *Nhe* I) and the insert (CC49 $V_H$ PCR product from pSCFVUHH treated with *Xho* I and *Nhe* I) are ligated in a 1:1 molar ratio, using 100 ng vector DNA (Stratagene kit) and used to transform *E. coli* AG1 competent cells (Stratagene) according to the manufacturer's directions. A colony with the correct plasmid DNA is picked as the pSC49FLAG clone.

Ligation of pATDFLAG Vector with PCR Amplified Human $V_H$ Inserts

The protocol for the ligation reaction is as follows:

| COMPONENT | AMOUNT |
|---|---|
| DNA vector:pATDFLAG *Xho* I/*Nhe* I | 2.5 µL |
| Hum $V_H$ (X) DNA inserts: *Xho* I/*Nhe* I | 6 µL |
| 10 mM ATP (Stratagene) | 2 µL |
| 10X buffer (Stratagene) | 2 µL |
| T4 DNA ligase (Stratagene) | 1 µL |
| ddH$_2$O | 6.5 µL |

DNA vector, ATP, 10X buffer and dd$H_2O$ are combined. DNA insert and T4 DNA ligase are then added. Ligation reactions are placed in a 4 L beaker containing $H_2O$ at 18 °C. The temperature of the water is gradually reduced by refrigeration at 4 °C overnight. This ligation reaction generates pHum4 $V_L$ - hum $V_H$ (X) (See Figure 29).

Transformation of *E. coli* AG1 with pHum4 $V_L$-Hum $V_H$ (X) Ligation Mix

Transformation of pATDFLAG into competent *E. coli* AG1 cells (Stratagene) is achieved following the supplier's protocol.

IBI MII Anti-FLAG Antibody Plate Assay

The first three steps, preparation of TAG-coated membranes, plating of bacterial membranes, and assembly of TAG and bacterial membranes, are the same as those described in the CC49-Biotin Competition Plate Assay.

After the 24 hour incubation at ambient temperatures, the membranes are washed with TTBS three times at 250 rpm for four minutes. The MII antibody (cat# IB13010, International Biotechnologies, Inc.) is then diluted with TBS to a concentration ranging from 10.85 µg/ml to 0.03 µg/ml. Ten millilters of the diluted antibody are added to each membrane. The membranes are then incubated for 1 hour at ambient temperatures and shaken on a rotary shaker at 70 rpm. After incubation, the MII antibody is removed and the membranes are washed three times at 250 rpm and ambient temperatures for 5 minutes. The final wash is removed and 20 milliters of a 1:2000 dilution of sheep anti-mouse horseradish peroxidase linked whole antibody (cat# NA931, Amersham) is prepared with TBS and added to each membrane. The membranes are again incubated for 1 hour at ambient temperatures and 70 rpm. Following incubation, the membranes are washed three times at 250 rpm and ambient temperature for 5 minutes each. Enzygraphic Webs (cat# IB8217051, International Biotechnologies, Inc.) are used to develop the membranes, according to the manufacturer's instructions. The membranes are then photographed.

Instead of seeing a clear zone on the developed membrane for a positive Hum4 $V_L$-$V_H$ (X) clone producing an SCFV that binds to TAG-72, (as seen with the competition screening assay) in this direct FLAG - detecting assay, a blue-purple spot is indicative of a colony producing a SCFV that has bound to the TAG-72 coated membrane. The advantage of using the FLAG system is that any Hum4 $V_L$ - $V_H$ SCFV combination that has bound to TAG-72 will be detected. Affinities can be measured

by Scatchard analysis (Scatchard (1949), *supra*) and specificity by immunohistochemistry. These canidates could then be checked for binding to a specific epitope by using the competition assay, *supra*, and a competing antibody or mimetic, if desired.

The present invention is not to be limited in scope by the cell lines deposited since the deposited embodiment is intended as two illustrations of one aspect of the invention and all cell lines which are functionally equivalent are within the scope of the invention. Indeed, while this invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications could be made therein without departing from the spirit and scope of the appended claims.

Example 6

Hum4 $V_L$ may also be used as a source of framework regions (FRs) for grafting the complementarity determining regions (CDRs) of the light chain variable region of an antibody, such as the $V_L$ of the TAG-72-specific antibody, CC49. When Hum4 $V_L$ FRs are used in a humanized variable region construct (*i.e.* comprising non-human CDRs), the FRs may also be modified by replacing one or more of their amino acids with, *e.g.*, murine, amino acids that may permit improvement in the functioning of the resulting antibody. Such an amino-acid-modified variable region is still considered a "humanized" region. An antibody or single chain antibody comprising a humanized light chain variable region having Hum4 $V_L$ FRs is herein termed a "humanized Hum4 $V_L$, $V_H$ antibody," *i.e.* any antibody or type of antibody in which the $V_L$(s) comprise (native or modified) Hum4 $V_L$ FRs and CDRs grafted thereon which are, or are derived from, non-human CDRs.

A humanized Hum4 $V_L$, $V_H$ antibody may use, as the heavy chain variable region(s) thereof, a $V_H$ which is entirely non-

human, chimeric (partly human), humanized, or entirely human. Specifically in regard to aTAG-72 humanized Hum4 $V_L$, $V_H$ antibodies based on CC49, the $V_H$ of such an antibody may be an entirely murine CC49 $V_H$, a chimeric CC49 $V_H$, or a humanized CC49 $V_H$. The procedures set forth below describe production of an embodiment of the lattermost type of CC49-based aTAG-72 humanized Hum4 $V_L$, $V_H$ antibody: "HuCC49*" a humanized CC49 monoclonal humanized Hum4 $V_L$, $V_H$ antibody having CC49 $V_L$ CDRs grafted upon Hum4 $V_L$ FRs and having a humanized CC49 $V_H$ region.

The specific light chain FRs chosen for use in humanizing the CC49 $V_L$ are derived from the light chain FRs of the human MAb, LEN (the LEN light chain being a human k Subgroup IV light chain). This particular light chain was selected from among the human k Subgroup IV light chain sequences reported in Kabat *et al.*, Sequences of Proteins of Immunological Interest (5th ed., 1991) (U.S. Department of Health and Human Services, NIH Publication No. 901-3242), based on the degree of similarity of its framework amino acid residues to certain framework residues of the native CC49 (nCC49) $V_L$ -- *i.e.* those residues potentially significant for maintenance of the combining site structure present in nCC49.

The decision as to which nCC49 amino acid residues were possibly significant, was itself based on study of a three-dimensional model of another antibody, McPC603, whose $V_L$ amino acids display identity to 95 of the 113 residues of the nCC49 $V_L$ (and identity to 69 of the 80 $V_L$ FR residues thereof). See E.A. Padlan, *Mol. Immunol.*, 31:169-217 (1994); however, the effects of specific amino acid residues and changes thereto are unpredictable. Based on this study, it was estimated that 43 of the nCC49 $V_L$ FR residues were possibly significant (see Fig. 32(A), asterisked residues), and the LEN $V_L$ was selected because its FR amino acids displayed identity in 36 of these 43 residues.

The same decision-making process was used to select the specific heavy chain FRs to be used in humanizing the nCC49 $V_H$.

These FRs are derived from the heavy chain FRs of the human MAb, 21/28¢CL, which was chosen based on a three-dimensional model of the antibody, 36-71. The $V_H$ amino acids of 36-71 display identity to 84 of the 115 residues of the nCC49 $V_H$, and identity to 71 of the 87 FR residues thereof. (See Padlan, *ibid.*) Based on the study of 36-71, it was estimated that 40 of the nCC49 $V_H$ residues were possibly significant (see Fig. 32(B), asterisked residues), and the 21/28¢CL $V_L$ was then selected because its FR amino acids displayed identity in 28 of these 40 residues.

Of the 7 remaining, non-identical "possibly significant" residues of the LEN $V_L$ FRs, and the 12 of the 21/28¢CL $V_L$ FRs, these residues were replaced with the corresponding amino acids of CC49 $V_L$ and CC49 $V_H$, respectively, so as to retain in the final, humanized antibody, all of the residues estimated as being "possibly significant." Thus, the humanized MAb, HuCC49*, was designed to comprise: 1) a humanized $V_L$ comprising the three $V_L$ CDRs of nCC49 and the residue-modified $V_L$ FRs of the human MAb, LEN; and 2) a humanized $V_H$ comprising the three $V_H$ CDRs of nCC49 and the residue-modified $V_H$ FRs of the human MAb, 21/28¢ CL. (See Fig. 32 which sets forth the humanization protocols for the CC49 $V_L$ and $V_H$ regions.)

Based on the resulting humanization protocols, nucleotide sequences were deduced from the amino acid sequence of each of the humanized $V_L$ and $V_H$ regions. The projected sequences were then refined by choosing codons for high frequency usage in the murine system and also by eliminating -- with the help of the programs FOLD and MAPSORT, (Devereux *et al.*, *Nucl. Acids Res.*, 12:387-395 (1984)) -- any self-annealing regions or any internal sites for the restriction endonucleases which were to be used for inserting the sequences into the appropriate vectors. The refined nucleotide sequences are shown in Figure 33.

For the generation of humanized $V_H$- and $V_L$- coding sequences, two sets of four 121- to 126-base-pair-long

oligonucleotides were synthesized. The four overlapping oligomers of a given set (depicted by long arrows in Figure 33) encompassed the entire refined nucleotide sequence of the humanized $V_H$ or $V_L$ gene on alternating strands. Double-stranded coding sequences were generated from the overlapping oligomers and then amplified, by the polymerase chain reaction (PCR), according to the following procedures.

First, four 20-base-pair-long amplification end primers were purchased (Midland Certified Reagent Co., Midland, TX) or synthesized (using a model 8700 DNA synthesizer Milligen/Bioresearch, Burlington, VT), and then these end primers were chromatographically purified (on Oligo-Pak columns from Milligen/Bioresearch). The sequences of these end primers were:

1. 5¢ $V_H$, coding: 5¢-CTAAGCTTCCACCATGGAG[?]-3¢;
2. 3¢ $V_H$, noncoding: 5¢-ATGGGCCCGTAGTTTTGGCG-3¢;
3. 5¢ $V_L$, coding: 5¢-GCAAGCTTCCACCATGGATA-3¢; and
4. 3¢ $V_L$, noncoding: 5¢-AGCCGCGGCCCGTTTCAGTT-3¢.

Both of the 5¢-primers carry a *Hind*III site, while the 3¢ $V_H$ primer has an *Apa*I site and the 3¢ $V_L$ primer carries a *Sac*II site at the flank.

PCR was carried out separately for each of the $V_L$ and $V_H$ coding sequences (data not shown), according to standard PCR reaction procedures. (Daugherty *et al.*, *Nucl. Acids, Res.*, 19:2471-2476 (1991).) To a final volume of 50 ml of PCR buffer -- containing 2.5 mM of each of the dNTPs and 2.5 units of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) -- 1 pmol each of the four overlapping oligomers and 50 pmol each of the two end primers were added. Three cycles of denaturation (1 min at 94°C), annealing (2 min at 55°C), and polymerization (2 min at 70°C) were followed by 17 additional cycles of denaturation (1 min at 94°C), annealing (2 min at 55°C), and polymerization (1 min at 72°C). This was followed by a final primer extension for 15 min at 72°C.

The DNA was extracted with phenol/chloroform and precipitated with ethanol. The amplified DNA was gel purified either as such or after treatment with the appropriate restriction endonucleases. Then the purified, PCR-generated copies of the DNA sequence encoding the humanized $V_L$ were cloned in the vector, pBluescript SK$^-$ (Stratagene, La Jolla, CA), while those for the humanized $V_H$ were separately cloned in the vector pCRII (a TA cloning vector designed for cloning PCR products, from Invitrogen, San Diego, CA) thereby generating pBSHuCC49*$V_L$ and pTAHuCC49*$V_H$, respectively. Each of the humanized variable regions was sequenced to check the fidelity of the PCR products.

After the fidelity of the PCR products was checked, eukaryotic expression vectors bearing genes comprising these variable region-encoding DNA sequences were constructed as illustrated in Figure 34. The expression vectors bear a gene for a selectable marker. This gene for the selectable marker is driven by the 5¢ long terminal repeat derived from M-MSV, while the human cytomegalovirus (HCMV) immediate early promoter drives the "target" gene, *i.e.* the HuCC49* light or heavy chain gene construct. A multiple cloning site is located immediately 3¢ to the HCMV promoter.

For the light chain of HuCC49*, pLNCXCC49Huk -- an expression construct of the cCC49 light chain -- was used as a source of DNA encoding the human k constant region. Taking advantage of an internal *Sac*II site and a *Cla*I site located 3¢ to the constant region DNA, a *SacII/Cla*I fragment encoding the human k constant region was excised therefrom. This fragment, together with the humanized $V_L$-encoding *Hind*III/*Sac*II fragment excised from pBSHuCC49*VL, was inserted directionally, by three-way ligation, between *Hind*III and *Cla*I sites in the retroviral expression construct pLNCX II, a retroviral vector. This vector is essentially the vector pLNCX, (Miller *et al.*, *Biotechniques*, 7:980-989 (1989)), obtained from Dr. D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA) and

modified by destroying an *EcoRI* site in the backbone of the vector while retaining another *EcoRI* site located 45 base pairs 5¢ to the neomycin resistance gene therein. pLNCX II is hereinafter referred to as pLNCX. Insertion of these two DNA fragments into pLNCX as indicated resulted in formation of pLNCXHuCC49HuK. (See Figure 34(A).)

For the heavy chain construct, an *ApaI/ClaI* DNA fragment encoding a human g1 constant region was excised from pLHCXCC49Hu$G_1$ -- an expression construct of the cCC49 heavy chain -- by taking advantage of an *ApaI* site in the $C_H1$ domain of the human g1 and a *ClaI* site located 3¢ to the g1 heavy chain. A *HindIII/ApaI* fragment encoding the humanized $V_H$ region was obtained from the construct pTAHuCC49*VH. Again, three-way ligation was used to directionally clone the two DNA fragments between the *HindIII* and *ClaI* sites of an expression vector, pLgpCX II. pLgpCX II is a retroviral vector derived from pLNCX II by replacing a 1.2-kb *BamHI* fragment carrying the neomycin resistance gene with a 0.7-kb *BglII/BamHI* fragment carrying the *Ecogpt* gene which had been excised from the vector pEE6HCMVgpt, (White *et al.*, *Protein Eng.*, 1:499-505 (1987)). The *Ecogpt* gene encodes xanthine-guanine phosphoribosyltransferase which confers resistance to mycophenolic acid in mammalia cells grown in culture medium supplemented with xanthine. pLgpCX II is hereinafter referred to as pLgpCX. Insertion of these two DNA fragments into pLgpCX as indicated resulted in formation of pLgpCXHuCC49Hu$G_1$. (See Figure 34(B).)

In order to express the HuCC49* MAb itself, the pLNCXHuCC49HuK and pLgpCXHuCC49Hu$G_1$ expression vector constructs were sequentially transfected into host cells as follows.

First, the expression construct, pLNCXHuCC49HuK, was electroporated into SP2/0 murine myeloma cells (of the SP2/0-Ag14 cell line, obtained as a gift from Dr. S. Morrison, University of California, Los Angeles), using the Cell-Porator

system (GIBCO BRL, Gaithersburg, MD). Electroporation was carried out as previously described (Slavin-Chiorini *et al.*, *Int. J. Cancer*, 53:97-103 (1993)), with minor modifications. Briefly, 40 mg of the *Pvu*I linearized DNA was added to a polypropylene electroporation chamber containing 1 x $10^7$ cells suspended in 1 ml of serum-free DMEM supplemented with 4.5 g/liter glucose. The cell/DNA mixture was placed in an ice-water bath and pulsed at 650 V/cm for 13 msec at a capacitance setting of 1600 mF. After keeping the cells on ice for 10 min, they were diluted in complete RPMI-1640 medium [RPMI-1640 containing 15% (v/v) heat-inactivated fetal calf serum, 2mM L-glutamine, 1mM sodium pyruvate, and 50 mg gentamicin/ml] and distributed in 24-well tissue culture plates (Costar, Cambridge, MA) at 1 x $10^5$ cells/wells. After incubation at 37°C in a 5% $CO_2$ incubator for 48 h, the medium was replaced with selection medium.

Selection medium consisted of complete RPMI-1640 containing 700mg/ml of active G418 (GIBCO BRL). After 2 weeks of selection in medium supplemented with G418, approximately 20% of the wells showed cell growth. Tissue culture supernatants from approximately 50% of the wells with cell growth were positive for human k chain reactivity, indicating that these cells were expressing the k light chain of HuCC49*.

Second, the expression construct pLgpCXHuCC49Hu$G_1$, was electroporated into a HuCC49* k chain-producing transfectant using the above-described procedure. However, after electroporation and incubation, the medium was instead replaced with a selection medium consisting of complete RPMI-1640 containing 1mg/ml mycophenolic acid (Sigma Chemical Co., St. Louis, MO), 250 mg/ml xanthine, and 15 mg/ml hypoxanthine (GIBCO BRL). After selection in this mycophenolic acid-containing medium, supernatants from two transfectants were reactive with a protein extract of TAG-72-positive LS-174T human colon carcinoma xenografts, indicating that these cells were expressing a whole aTAG-72 antibody.

In addition, no reactivity was observed to a protein extract of TAG-72-negative A375 human melanoma xenografts (the A375 human melanoma cell line being obtained from Dr. S. Aaronson, National Cancer Institute, NIH, Bethesda, MD), thereby indicating that these two transfectants were expressing an antibody specific for TAG-72. The transfectant that secreted a higher titer of TAG-72-reactive Ig was cloned by limiting dilution, and the subclone that produced the highest titer -- designated HuCC49* -- was adapted for growth in serum- and protein-free medium (PFHM-II, Gibco BRL).

In order to assess the purity of the HuC49* antibody, and to characterize its mobility relative to that of chimeric CC49 (cCC49), SDS-PAGE analysis was performed under reducing and non-reducing conditions. Quantities of the HuCC49* antibody sufficient for these analyses were obtained by growing the above-selected HuCC49* clone in protein-free hybridoma medium PFHM-II (Gibco BRL), followed by isolation from the tissue culture supernatants via protein G affinity chromatography and concentration of the harvested antibody as follows: 1) the tissue culture supernatants were applied to a recombinant protein G (Gibco BRL) agarose column; 2) the bound protein was eluted from the column using 0.1 M glycine hydrochloride buffer, pH 2.6; 3) the pH of the eluted material was immediately adjusted to 7.0 using 1.0 M Tris buffer, pH 8.0; 4) the pH 7.0 material was dialyzed against phosphate-buffered saline (PBS), pH 7.4; and 5) the dialyzed material was concentrated using an immersible-CX-30 ultrafilter (Millipore, Bedford, MA).

The HuCC49* protein concentration was determined using a Bio-Rad Microassay procedure, (M.M. Bradform, *Anal. Biochem.*, 72:248-254 (1976)), or by the method of Lowry *et al.* (*J. Biol. Chem.*, 193:265-275 (1951)). Approximately 1 mg of HuCC49* was recovered per ml of the tissue culture supernatants. cCC49 was purified from tissue culture supernatant using high-

performance liquid chromatography and the protein concentration was likewise determined.

PAGE analyses of cCC49 and HuCC49* were performed on precast 4-20% SDS-polyacrylamide Tris-glycine gels (Novex, San Diego, CA) with and without denaturation with 2-mercaptoethanol. Proteins on the gel were visualized by staining with Coomassie Brilliant blue R250 according to the method of U.K. Laemmli (*Nature* (London), 227:680-685 (1970)).

The gel profiles under non-reducing conditions showed that the HuCC49* MAb (Fig. 35(A), lane 2) has virtually identical mobility to that of cCC49 (Fig. 35(A), lane 1), which has a molecular mass of approximately 160 kDa. Under reducing conditions, the HuCC49* MAb (Fig. 35(B), lane 2) yielded two protein bands of approximately 25-28 and 50-55 kDa. This is consistent with the molecular masses of the heavy and light chains of cCC49 (Fig. 35(B), lane 1).

In order to better characterize HuCC49* relative to cCC49 and nCC49, purified HuCC49*, cCC49, and nCC49 were obtained and radio-labeled for use in further analysis by PAGE, HPLC, and immunoreactivity studies (the development and reactivity of nCC49 has been previously described by Kuroki *et al.*, *Cancer Res.*, 48:4588-4596 (1988)). Thus, these three antibodies were labeled with $Na^{125}I$ or $Na^{131}I$ using the Iodo-Gen (Pierce, Rockford, IL) method of Colcher *et al.* (*Cancer Res.*, 43:736-742 (1983)). The iodination protocol resulted in $^{125}I$-labeled cCC49, 125I-labeled nCC49, and $^{131}I$-labeled HuCC49* with specific activities of 2-5 mCi/mg.

These three radioiodinated antibodies were evaluated by SDS-PAGE analysis under non-reducing and reducing conditions. The radioiodinated MAbs were detected by autoradiography using Kodak XAR X-ray film (Rochester, NY) and Lightning Plus intensifying screens (E.I. DuPont deNemours & Co., Wilmington, DE). Molecular weight profiles, similar to those described for the unlabeled purified MAbs, were observed.

The integrity of each of the radioiodinated CC49 molecules was then evaluated by HPLC size-exclusion chromatography. The HPLC analyses were performed using a Sepherogel-TSK 2000 SW, 0.75 x 30 cm column (Beckman Instruments, Inc., Berkeley, CA) equilibrated in 67 mM sodium phosphate containing 100 mM KCl, pH 6.8. Samples (250,000 cpm in 25 ml) were applied and eluted from the column at a flow rate of 0.5 ml/min. The radioactivity was measured in a flow-through gamma scintillation counter (Model 170; Beckman Instruments, Inc.). Each of $^{131}$I-labeled HuCC49*, $^{125}$I-labeled cCC49, and $^{125}$I-labeled nCC49 eluted from the column at 29 min. as a distinct species (see Figure 36(A) and (B): data not shown for $^{125}$I-labeled nCC49).

Finally, the immunoreactivities of the radiolabeled antibodies were assessed by a modification of a method previously described by Schott *et al.*(*Cancer Res.*, 52:6413-5417 (1982)), using bovine submaxillary mucin (BSM). BSM was immobilized onto solid support gel beads (Reacti-Gel HW65F from Pierce, Rockford, IL) as detailed by Jonson *et al.*(*Cancer Res.*, 46:850-857 (1986)), at a ratio of 2 mg BSM to 1 ml of wet-packed gel, and the TAG-72-positive BSM beads were used to perform the radioimmuno-reactivity assay. 50 ml of wet-packed BSM beads was placed in each tube of (multiple sets of) three pairs of 1.5 ml microfuge tubes. The radiolabeled antibodies were diluted to 23 nCi in 1 ml of 1% bovine serum albumin (BSA) in PBS. The radiolabeled antibodies were then added to the duplicate tubes, counted in a gamma scintillation counter, and incubated for 2 h at room temperature with end-over-end rotation. The BSM beads were then pelleted at 800´g for 5 min, and the beads in each tube were washed twice with 1 ml of 1% BSA in PBS. The radioactivity remaining in each tube was measured and the total percent bound to the BSM beads was calculated. The percent bound for each of the radiolabeled Ig forms was greater than 85, while the percent bound for a nonspecific antibody was typically <10%. Approximately 85% of the $^{131}$I-labeled HuCC49* and 90% of the $^{125}$I-labeled cCC49 MAbs

bound to BSM beads, thus indicating the immunoreactivity of the HuCC49* and cCC49 MAbs.

Next, the relative affinity constants ($K_a$) of HuCC49*, and cCC49 and nCC49, were determined using a competition radioimmunoassay (RIA) technique. Competition RIAs were performed using $^{125}$I-labeled nCC49 as the radiolabeled antibody and BSM as the target antigen, according to the method of Hand *et al.* (*Cancer Immunol. Immunother.*, 35:165-174 (1992)). In these RIAs, $^{125}$I-labeled nCC49 was used to compete for the binding of each of the three unlabeled competitor antibodies bound to the TAG-72-positive BSM. The percentage of radiolabeled MAb bound to antigen (% bound), compared to a buffer control was calculated.

As shown in Figure 37, all three CC49 MAbs were able to completely inhibit the binding of the $^{125}$I-labeled nCC49 to TAG-72, although the level of competition observed with the HuCC49* MAb differed from that of the nCC49 and cCC49 MAbs. Approximately 45 mg of the HuCC49* was required for 50% competition, as compared with 1.5 and 2.0 mg of the nCC49 and cCC49 MAbs, respectively. No competition was observed when control murine IgG (MOPC-21, an $IgG_1$ murine myeloma protein obtained from Organon Technika, Durham, NC) and control human IgG (purified IgG obtained from Jackson Immuno-Research, West Grove, PA) were used as competitors.

The $K_a$s of HuCC49*, cCC49, and nCC49 were determined using the method of G. Scatchard (*Ann. NY Acad. Sci.*, 51:660-668 (1949)). The relative affinity constant of the cCC49 MAb was found to be $1.2 \times 10^8$ $M^{-1}$ and that of the nCC49 MAb was found to be $1.8 \times 10^8$ $M^{-1}$, while the $K_a$ of HuCC49* was $6.0 \times 10^7$ $M^{-1}$, *i.e.* approximately 2- to 3-fold less than those of the nCC49 and cCC49 MAbs, respectively.

Studies were then initiated to compare the plasma clearance and biodistribution of HuCC49* with that of cCC49. It has previously been shown that the pharmacokinetics of nCC49 and cCC49 in mice differ greatly, with cCC49 clearing

more rapidly. This has been shown to affect *in vivo* tumor targeting. Thus, HuCC49* and cCC49 were compared in pharmacokinetics and *in vivo* targeting studies as follows. Each of 5 athymic (nu/nu) mice received one i.v. injection containing a mixture of 0.94 mCi/mouse of $^{125}$I-labeled cCC49 and 0.98 mCi/mouse of $^{131}$I-labeled HuCC49*. Blood samples were collect at specified time intervals via the tail vein into 10-ml capillary tubes (Drummon, Broomall, PA). The amount of $^{131}$I and $^{125}$I in the plasma was determined and normalized to account for differences in the rates of decay of the radionuclides. The percentage of the injected dose of each radionuclide remaining the plasma was then calculated.

The data from this experiment was used to determine the half-lives of the antibodies in plasma, using a curve-fitting program. The $t_{1/2}$a and $t_{1/2}$b of HuCC49* were 4.2 and 149 h, respectively. These values are comparable to the $t_{1/2}$a and $t_{1/2}$b values for cCC49, *i.e.* 4.7 and 139 h, respectively. Statistical analysis was also performed on this plasma clearance data using a 2-tailed paired Student's *t* test with n = 5 and 4 degrees of freedom. Figure 38 shows that both MAbs have similar blood clearance patterns with approximately 74% of the radiolabeled MAb dose clearing the blood at 24 h and 83% by 72h.

Experiments were then conducted to assess the biodistribution of these antibodies in order to compare the ability of HuCC49* to localize to human tumor xenografts with that of cCC49. The biodistribution of the HuCC49* MAb was compared with that of the cCC49 MAb as follows. Female athymic (nu/nu) mice bearing TAG-72-positive tumor xenografts were produced according a method previously described by Colcher *et al.* (*Cancer Res.*, 43:736-742 (1983)), using cells from the LS-174T human colon adenocarcinoma cell line (described by Rutzki *et al.*, *In Vitro*, 12:180-191 (1976)) which was obtained from the American Type Culture Collection (Rockville, MD).

These tumor-bearing mice were injected in the tail vein with a mixture containing approximately 0.94 mCi/mouse of $^{125}I$-labeled cCC49 and 0.98 mCi/mouse of $^{131}I$-labeled HuCC49*. Blood, tumor, and all major organs were collected and weighted (5 mice per time point). Radioactivity was measured in a gamma scintillation counter and radioactive decay was counted. The percentage of the injected dose per gram (% ID/g) for each organ was determined and the radiolocalization indices (%ID/g in tumor divided by the %ID/g in normal tissue) were calculated. Statistical analysis was also done for the biodistribution data using a 2-tailed paired Student's *t* test with n = 5 and 4 degrees of freedom.

No statistically significant difference was observed between the %ID/g of either MAb to tumors or tissues collected at any time point (Table 2). Both antibodies showed tumor localization by 24 h; by 96 h, when there was <2% of the injected dose per ml of blood, the %ID/g in tumor was 22.6% for HuCC49* and 19.5% for cCC49 (Table 2). No specific uptake of either radiolabeled antibody was observed in any normal tissue tested. As shown in Table 3, the radiolocalization indices (RIs) (%ID/g in tumor divided by the %ID/g in normal tissue) of the two MAbs were not appreciably different for any tumor:normal tissue ratio. Thus, these data indicate that the HuCC49* and cCC49 MAbs have similar tumor-targeting properties.

**TABLE 2. Radiolocalization of $^{131}I$-Labeled HuCC49* and $^{125}I$-Labeled CCC49 in Athymic Mice Bearing LS-174T Tumors (%ID/g)[a]**

| Antibody, Organ | %ID/g | | | | |
|---|---|---|---|---|---|
| | 24h | 48h | 72h | 96h | 168h |
| HuCC49* | | | | | |
| Tumor | 15.4±7.2[b] | 22.8±17.1 | 16.1±12.8 | 22.6±5.3 | 12.9±12.4 |
| Blood | 5.1±1.4 | 4.1±2.9 | 2.2±1.8 | 1.6±0.8 | 0.8±0.7 |
| Liver | 3.3±1.6 | 2.7±0.9 | 1.5±0.8 | 1.6±0.2 | 0.6±0.3 |

| | | | | | |
|---|---|---|---|---|---|
| Spleen | 5.7±3.9 | 4.7±0.9 | 2.7±1.6 | 4.6±0.8 | 3.3±2.5 |
| Kidneys | 1.5±0.4 | 1.1±0.4 | 0.8±0.5 | 0.6±0.1 | 0.4±0.2 |
| Lungs | 2.0±0.3 | 2.0±1.2 | 1.0±0.7 | 1.0±0.4 | 0.4±0.3 |
| cCC49 | | | | | |
| Tumor | 16.1±7.5 | 23.4±19.0 | 14.9±12.0 | 10.5±6.0 | 12.3±11.3 |
| Blood | 4.5±1.6 | 3.6±2.8 | 1.7±1.4 | 1.1±0.7 | 0.6±0.4 |
| Liver | 3.8±1.8 | 3.0±0.8 | 1.4±0.9 | 1.3±0.2 | 0.4±0.3 |
| Spleen | 8.5±5.5 | 6.7±1.2 | 3.5±2.5 | 7.5±2.7 | 4.8±3.8 |
| Kidneys | 1.6±0.4 | 1.0±0.5 | 0.6±0.4 | 0.4±0.1 | 0.3±0.1 |
| Lungs | 2.1±0.3 | 1.9±1.2 | 0.8±0.5 | 0.8±0.4 | 0.4±0.2 |

[a] Athymic mice (5 per group) bearing LS-174T human colon carcinoma xenografts were injected i.v. with a mixture containing approximately 10mCi of each radiolabeled MAb, and sacrificed at the indicated times.

[b] Values represent the mean %ID/g±SD of samples from 5 mice.

**TABLE 3. Radiolocalization of $^{131}$I-Labeled HuCC49* and $^{125}$I-Labeled cCC49 in Athymic Mice Bearing LS-174T Tumors (Radiolocalization Index)[a]**

| Antibody, Organ | Radiolocalization Index[b] | | | | |
|---|---|---|---|---|---|
| | 24h | 48h | 72h | 96h | 168h |
| HuCC49* | | | | | |
| Blood | 3.1±1.1[c] | 6.8±4.1 | 8.6±3.7 | 16.7±5.5 | 24.7±23.6 |
| Liver | 5.4±3.3 | 9.2±7.4 | 9.2±3.9 | 14.1±3.9 | 21.8±14.3 |
| Spleen | 3.6±2.7 | 5.3±4.5 | 5.4±2.9 | 5.0±1.6 | 5.1±5.1 |
| Kidneys | 10.2±4.8 | 19.5±8.6 | 18.2±5.5 | 37.7±3.2 | 30.6±20.4 |
| Lungs | 7.7±3.1 | 11.4±4.3 | 14.6±3.2 | 25.5±7.3 | 36.6±27.6 |
| cCC49 | | | | | |
| Blood | 3.8±1.4 | 9.1±7.0 | 12.1±7.1 | 21.0±6.9 | 27.3±23.8 |
| Liver | 4.7±2.6 | 8.2±6.6 | 9.3±2.6 | 14.6±3.9 | 25.8±17.0 |
| Spleen | 2.4±1.6 | 3.5±2.5 | 4.4±2.4 | 2.9±1.4 | 3.6±3.4 |
| Kidneys | 10.2±4.7 | 20.4±8.0 | 21.5±5.3 | 46.1±7.5 | 40.6±26.9 |
| Lungs | 7.9±3.5 | 11.9±4.4 | 16.0±3.7 | 26.6±9.1 | 32.2±22.7 |

[a] Athymic mice (5 per group) bearing LS-174T human colon carcinoma xenografts were injected i.v. with a mixture containing approximately 1.0μCi of each radiolabeled MAb, and sacrificed at the indicated times.

[b] The radiolocalization index is the ratio of the %ID/g of the tumor tissue to the %ID/g of the normal tissue.

[c] Values represent the radiolocalization index ±SD of samples from 5 mice.

Other embodiments of the invention will be apparent to those skilled in the art from a consideration of this specification or practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope and spirit of the invention being indicated by the following claims.

What is claimed is:

1. A humanized or composite anti-TAG-72 antibody or anti-TAG-72 antibody fragment which comprises a CDR-grafted light chain having non-human CDRs grafted to a human subgroup IV kappa light chain.

2. The humanized antibody or humanized antibody fragment of Claim 1 wherein the non-human CDRs are obtained from a murine antibody.

3. The humanized antibody or humanized antibody fragment of Claim 2 wherein said murine antibody is CC49, CC83, CC46, CC92, CC30, or CC11.

4. The humanized antibody or humanized antibody fragment of Claim 3 wherein said murine antibody is CC49.

5. The humanized antibody or humanized antibody fragment of Claim 4 wherein the antibody or fragment comprises the variable heavy and/or light regions encoded by the humanized variable heavy and light sequences contained in Figure 14.

6. The humanized antibody or humanized antibody fragment of Claim 5 wherein said humanized antibody is expressed by ATCC HB-12404 and said humanized antibody fragment has an amino acid sequence identical to that of a constituent part of the antibody expressed by ATCC HB-12404.

7. A nucleic acid sequence from which may be expressed the humanized antibody or humanized antibody fragment according to Claim 2.

8. A vector from which may be expressed the humanized antibody or humanized antibody fragment according to Claim 2.

9. The vector according to Claim 8 wherein said vector is a bare nucleic acid segment, a carrier-associated nucleic acid segment, a nucleoprotein, a plasmid, a virus, a viroid, or a transposable element.

10. A composition suitable for the treatment or *in vivo* or *in vitro* detection of cancer comprising, respectively, a therapeutically effective or a diagnostically effective amount of a humanized antibody or humanized antibody fragment according to Claim 2.

11. The composition of Claim 10 wherein said humanized antibody or humanized antibody fragment is, directly or indirectly, associated with or linked to an effector moiety having therapeutic activity, and said composition is suitable for the treatment of cancer.

12. The composition of Claim 11 wherein said effector moiety is a radionuclide, therapeutic enzyme, anti-cancer drug, cytokine, cytotoxin, or anti-proliferative agent.

13. The composition of Claim 12 wherein the radionuclide is selected from the group consisting of $^{47}Sc$, $^{67}Cu$, $^{67}Ga$, $^{90}Y$, $^{99m}Tc$, $^{105}Rh$, $^{111}In$, $^{123}I$, $^{125}I$, $^{131}I$, $^{153}Sm$, $^{177}Lu$, $^{186}Re$, $^{188}Re$, $^{211}At$, $^{212}Bi$, and chelates thereof.

14. The composition of Claim 10 wherein said humanized antibody or humanized antibody fragment is, directly or indirectly, associated with or linked to a a detectable label, and the composition is suitable for detection of cancer.

15. The composition of Claim 14 wherein the detectable label is a radionuclide or an enzyme.

16. The composition of Claim 15 wherein the radionuclide is selected from the group consisting of $^{67}Cu$, $^{67}Ga$, $^{99m}Tc$, $^{105}Rh$, $^{111}In$, $^{123}I$, $^{125}I$, $^{131}I$, $^{145-158}Eu$ (all Europeum isotopes), $^{153}Sm$, $^{148-161}Tb$ (all Terbium isotopes), $^{166}Ho$, $^{177}Lu$, $^{186}Re$, $^{188}Re$, and chelates thereof.

17. A method for *in vivo* treatment of a mammal having a TAG-72-expressing cancer which comprises administering to the mammal a therapeutically effective amount of a composition according to Claim 10.

18. A method of *in vitro* detection of TAG-72-expressing cancer cells which comprises contacting the cancer cells with a composition according Claim 10.

19. method of *in vivo* detection of TAG-72-expressing cancer cells in a mammal which comprises administering to the mammal a diagnostically effective amount of a composition according to Claim 10.

20. The method of Claim 19 wherein said detection is *in vivo* tumor imaging.

21. A method of treatment of cancer comprising (i) intravenously administering a radionuclide-labeled antibody composition of Claim 10, (ii) thereafter, during surgery, detecting tumor cells using a radionuclide activity probe, and (iii) thereafter removing the detected tumor cells by excision.

22. A method for *in vivo* diagnosis of cancer which comprises the steps of (a) administering to an mammal a pharmaceutically effective amount of the composition of Claim 10, (b) allowing sufficient time for the imaging marker-antibody conjugates of said composition to become specifically localized upon at least one cancer cell, and (b) detecting the imaging marker *in vivo* at a site where said conjugate has become localized.

23. A method of intraoperative therapy which comprises the steps of (a) performing upon an mammal having at least one cancer cell the method of Claim 21 and (b) excising said cancer.

24. The method of Claim 23, wherein the radionuclide is $^{125}I$ or $^{131}I$.

25. A commercial package comprising a composition

according to Claim 10 as active ingredient together with instructions for use thereof to treat or detect cancer wherein the composition is reconstituted prior to said use.

ABSTRACT

Novel composite and humanized anti-TAG-72 monoclonal antibodies, antibody fragments, and derivatives thereof using human subgroup IV kappa light chain framework regions.

Fig. 1

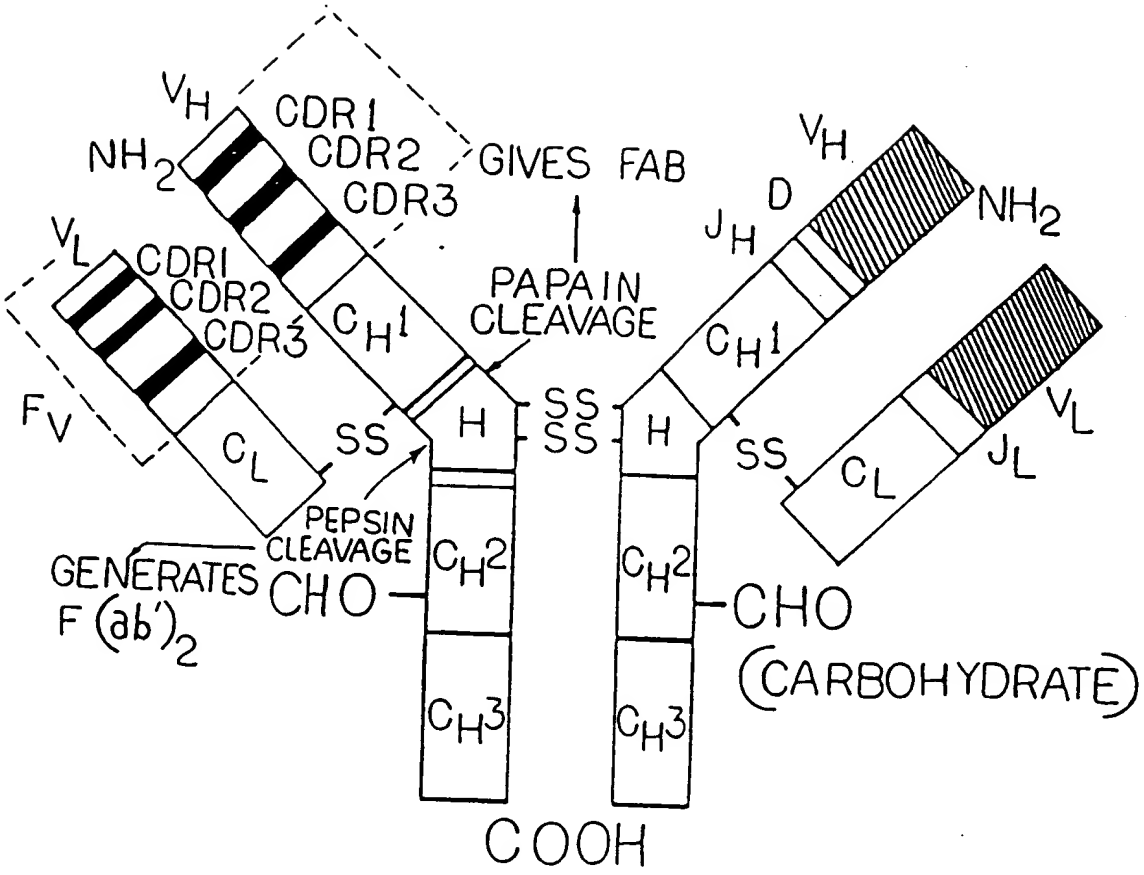

$V_H$
CDR1
CDR2
CDR3
$NH_2$
GIVES FAB
$V_L$
CDRI
CDR2
CDR3
$C_H1$
PAPAIN CLEAVAGE
$V_H$
D
$J_H$
$NH_2$
$C_H1$
$V_L$
$J_L$
$F_V$
$C_L$
SS
H
SS
SS
H
SS
$C_L$
PEPSIN CLEAVAGE
GENERATES $F(ab')_2$
CHO
$C_H2$
$C_H2$
CHO
(CARBOHYDRATE)
$C_H3$
$C_H3$
COOH

FIG.2

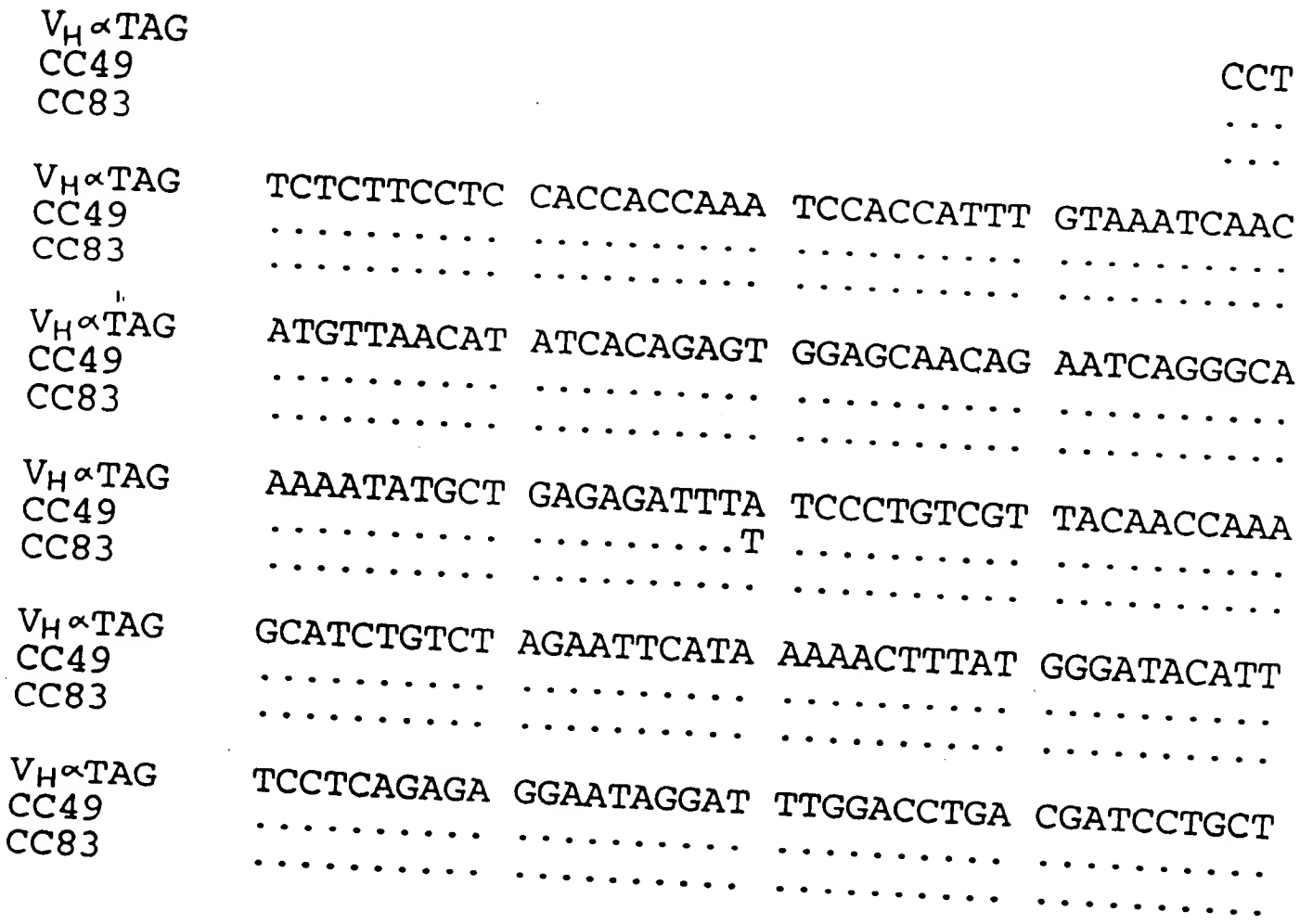

$V_H\alpha$TAG
CC49
CC83
CCT
...
...
$V_H\alpha$TAG
CC49
CC83
TCTCTTCCTC CACCACCAAA TCCACCATTT GTAAATCAAC
..... ..... ..... .....
..... ..... ..... .....
$V_H\alpha$TAG
CC49
CC83
ATGTTAACAT ATCACAGAGT GGAGCAACAG AATCAGGGCA
..... ..... ..... .....
..... ..... ..... .....
$V_H\alpha$TAG
CC49
CC83
AAAATATGCT GAGAGATTTA TCCCTGTCGT TACAACCAAA
..... .....T ..... .....
..... ..... ..... .....
$V_H\alpha$TAG
CC49
CC83
GCATCTGTCT AGAATTCATA AAAACTTTAT GGGATACATT
..... ..... ..... .....
..... ..... ..... .....
$V_H\alpha$TAG
CC49
CC83
TCCTCAGAGA GGAATAGGAT TTGGACCTGA CGATCCTGCT
..... ..... ..... .....
..... ..... ..... .....

FIG. 2 (CONT.)

| | | | | |
|---|---|---|---|---|
| $V_H$αTAG | GCCCGAGCCA | TGTGATGACA | GTTCTTCTCC | AGTTGAACTA |
| CC49 | .......... | .......... | .......... | .......... |
| CC83 | .......... | .......... | .......... | .......... |
| $V_H$αTAG | GGTCCTTATC | TAAGAAATGC | ACTGCTCATG | AATATGCAAA |
| CC49 | .......... | .......... | .......... | .......... |
| CC83 | .......... | .......... | .......... | .......... |
| $V_H$αTAG | TCACCCGAGT | CTATGGCAGT | AAATACAGAG | ATGTTCATAC |
| CC49 | .......... | .......... | .......... | .......... |
| CC83 | .......... | .......... | .......... | .......... |
| $V_H$αTAG | CATAAAAACA | ATATATGATC | AGTGTCTTCT | CCGCTATCCC |
| CC46 | | ......... | .......... | .......... |
| CC49 | .......... | ....G..... | .......... | .......... |
| CC83 | .......... | .......... | .......... | .......... |
| CC92 | | | | ........ |

FIG. 2 (CONT.)

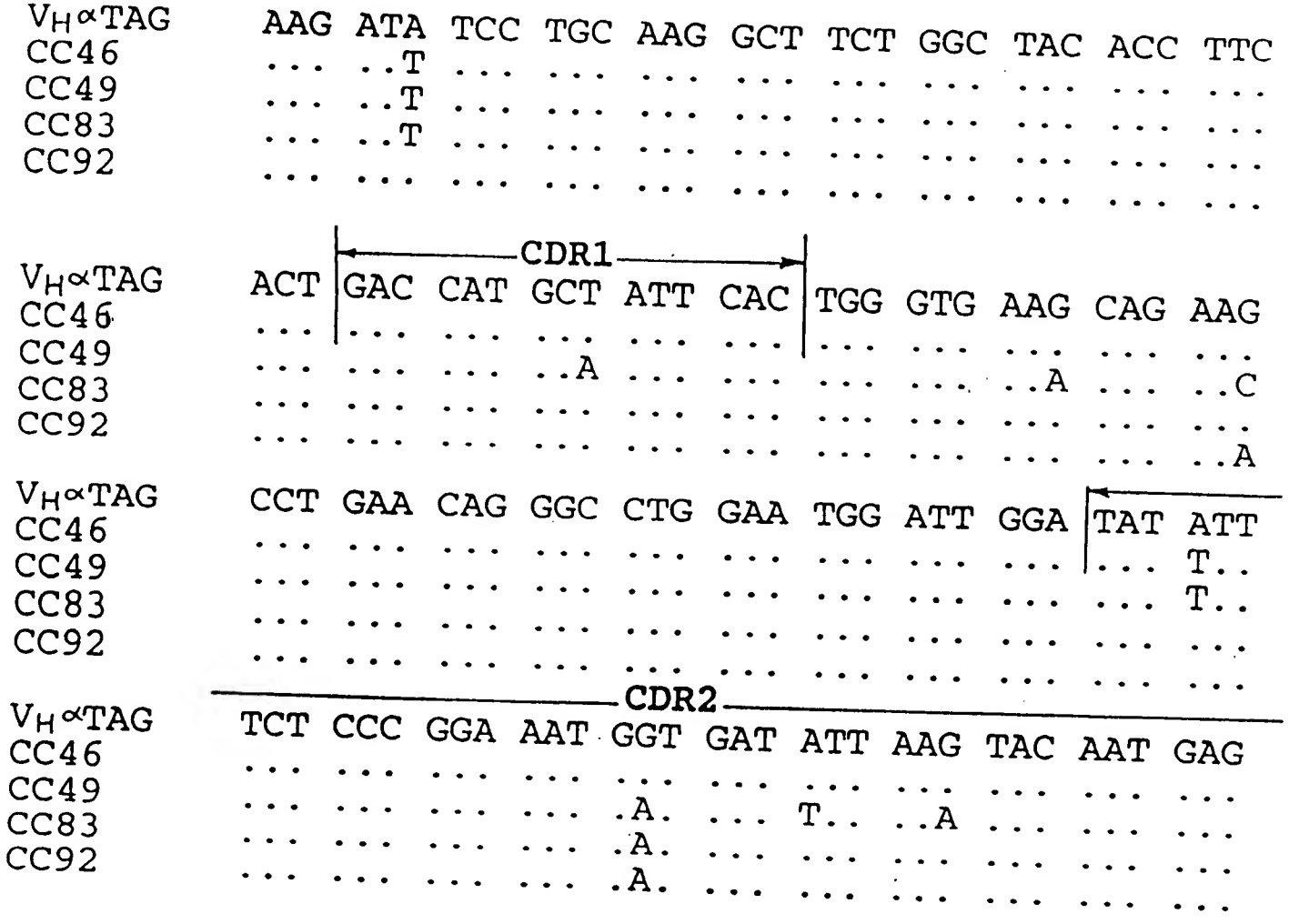

VH∝TAG AAG ATA TCC TGC AAG GCT TCT GGC TAC ACC TTC
CC46 ... ..T ... ... ... ... ... ... ... ... ...
CC49 ... ..T ... ... ... ... ... ... ... ... ...
CC83 ... ..T ... ... ... ... ... ... ... ... ...
CC92 ... ... ... ... ... ... ... ... ... ... ...
CDR1
VH∝TAG ACT GAC CAT GCT ATT CAC TGG GTG AAG CAG AAG
CC46 ... ... ... ... ... ... ... ... ... ... ...
CC49 ... ... ... ..A ... ... ... ... ..A ... ..C
CC83 ... ... ... ... ... ... ... ... ... ... ...
CC92 ... ... ... ... ... ... ... ... ... ... ..A
VH∝TAG CCT GAA CAG GGC CTG GAA TGG ATT GGA TAT ATT
CC46 ... ... ... ... ... ... ... ... ... ... ...
CC49 ... ... ... ... ... ... ... ... ... ... T..
CC83 ... ... ... ... ... ... ... ... ... ... T..
CC92 ... ... ... ... ... ... ... ... ... ... ...
CDR2
VH∝TAG TCT CCC GGA AAT GGT GAT ATT AAG TAC AAT GAG
CC46 ... ... ... ... ... ... ... ... ... ... ...
CC49 ... ... ... ... .A. ... T.. ..A ... ... ...
CC83 ... ... ... ... .A. ... ... ... ... ... ...
CC92 ... ... ... ... .A. ... ... ... ... ... ...

FIG. 2 (CONT.)

| | |
|---|---|
| $V_H\alpha$TAG | GCCTGAGGTG ACAAAGATAT CACCTTTGGC TTTCCACAG |
| CC46 | ..... ..... ..... ..... ..... |
| CC49 | ..... .....G. ..... .....G..... |
| CC83 | ..... ..... ..... ..... ..... |
| CC92 | ..... ..... ..... ..... ..... |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $V_H\alpha$TAG | GT | GTC | CAC | TCC | CAG | GTT | CAG | CTG | CAG | CAG | TCT |
| CC46 | .. | ... | ... | ... | ..A | T.. | ... | ..A | ... | ... | ... |
| CC49 | .. | ... | ... | ... | ... | ... | ... | T.. | ... | ... | ... |
| CC83 | .. | ... | ... | ... | ... | ... | ... | T.. | ... | ... | ... |
| CC92 | .. | ... | ... | ... | ... | ... | ..A | ... | ... | ... | ... |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $V_H\alpha$TAG | GAC | GCT | GAG | TTG | GTG | AAA | CCT | GGG | GCT | TCA | GTG |
| CC46 | ... | ... | ... | ... | ... | .G. | ... | ... | ... | ... | ... |
| CC49 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| CC83 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| CC92 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |

FIG. 2 (CONT.)

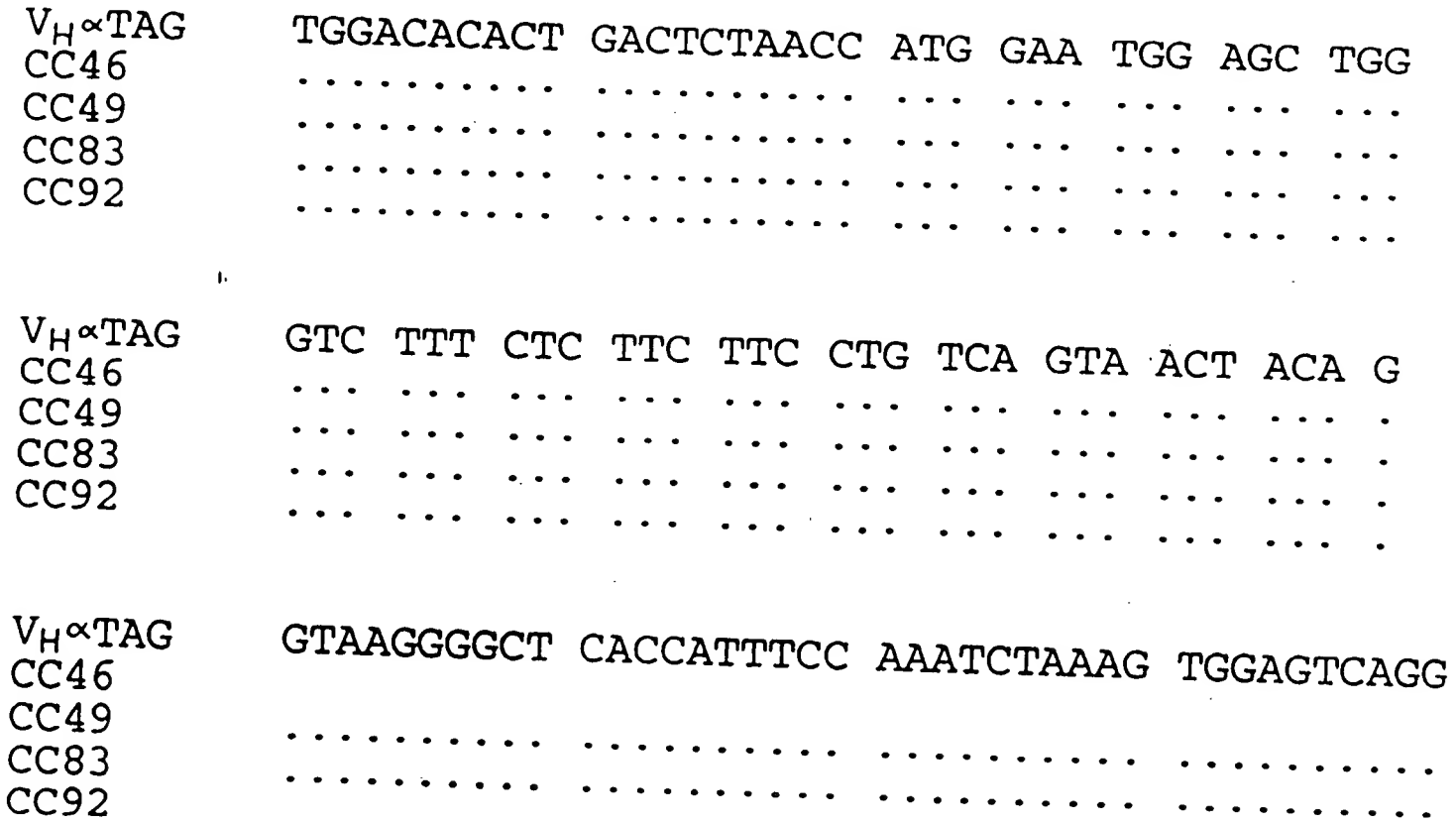

$V_H$∝TAG
TGGACACACT GACTCTAACC ATG GAA TGG AGC TGG
CC46
CC49
CC83
CC92
$V_H$∝TAG
GTC TTT CTC TTC TTC CTG TCA GTA ACT ACA G
CC46
CC49
CC83
CC92
$V_H$∝TAG
GTAAGGGGCT CACCATTTCC AAATCTAAAG TGGAGTCAGG
CC46
CC49
CC83
CC92

FIG. 2 (CONT.)

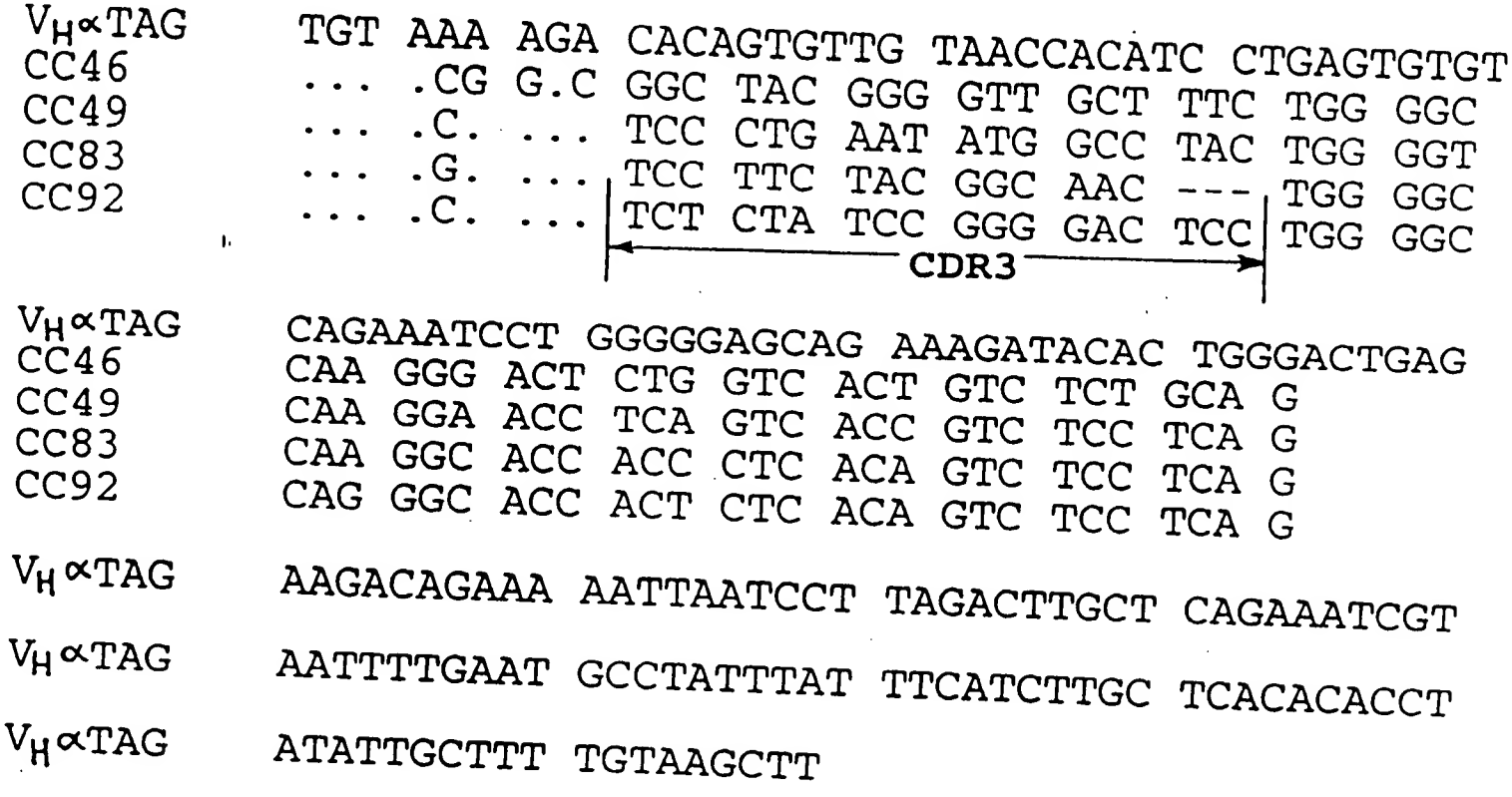

VH∝TAG TGT AAA AGA CACAGTGTTG TAACCACATC CTGAGTGTGT
CC46 ... .CG G.C GGC TAC GGG GTT GCT TTC TGG GGC
CC49 ... .C. ... TCC CTG AAT ATG GCC TAC TGG GGT
CC83 ... .G. ... TCC TTC TAC GGC AAC --- TGG GGC
CC92 ... .C. ... TCT CTA TCC GGG GAC TCC TGG GGC
CDR3
VH∝TAG CAGAAATCCT GGGGGAGCAG AAAGATACAC TGGGACTGAG
CC46 CAA GGG ACT CTG GTC ACT GTC TCT GCA G
CC49 CAA GGA ACC TCA GTC ACC GTC TCC TCA G
CC83 CAA GGC ACC ACC CTC ACA GTC TCC TCA G
CC92 CAG GGC ACC ACT CTC ACA GTC TCC TCA G
VH∝TAG AAGACAGAAA AATTAATCCT TAGACTTGCT CAGAAATCGT
VH∝TAG AATTTTGAAT GCCTATTTAT TTCATCTTGC TCACACACCT
VH∝TAG ATATTGCTTT TGTAAGCTT

FIG.3

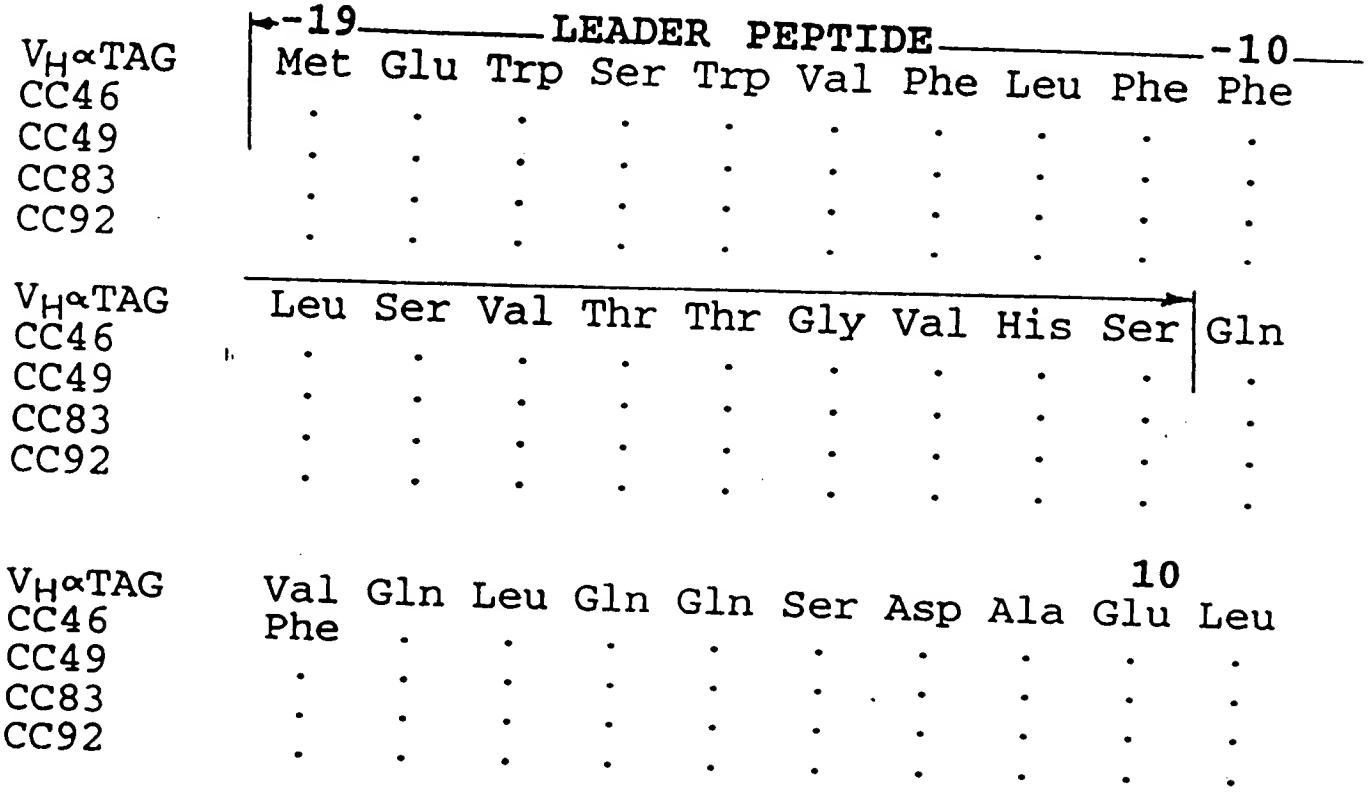

-19
LEADER PEPTIDE
-10
VHαTAG
CC46
CC49
CC83
CC92
Met Glu Trp Ser Trp Val Phe Leu Phe Phe
Leu Ser Val Thr Thr Gly Val His Ser Gln
Val Gln Leu Gln Gln Ser Asp Ala Glu Leu
Phe
10

FIG. 3 (CONT.)

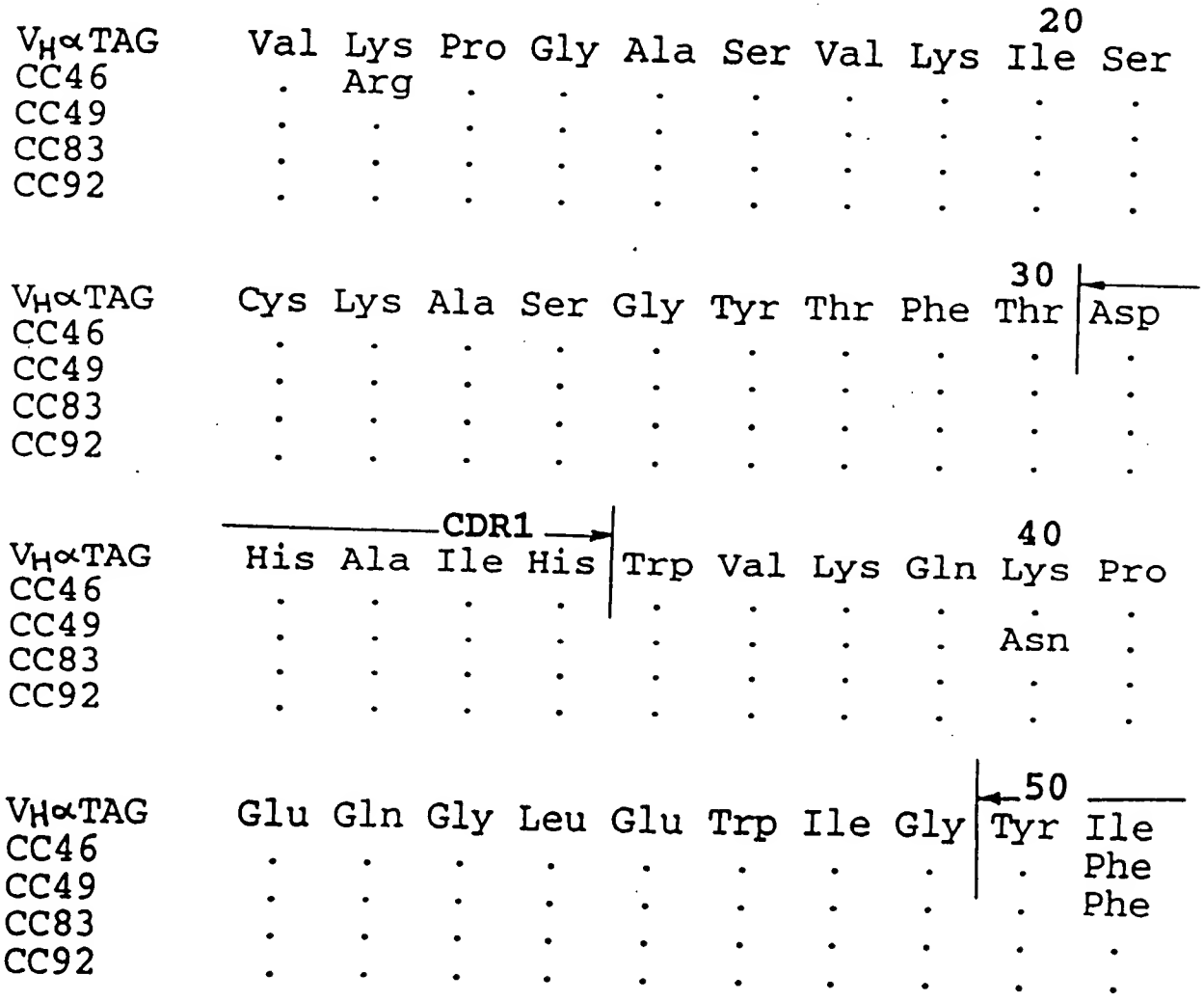

VHαTAG
CC46
CC49
CC83
CC92
Val Lys Pro Gly Ala Ser Val Lys Ile Ser
20
Arg
Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp
30
His Ala Ile His Trp Val Lys Gln Lys Pro
CDR1
40
Asn
Glu Gln Gly Leu Glu Trp Ile Gly Tyr Ile
50
Phe
Phe

FIG. 3 (CONT.)

| $V_H$αTAG | Ser | Pro | Gly | Asn | Gly | Asp | Ile | Lys | Tyr | Asn |
|---|---|---|---|---|---|---|---|---|---|---|
| CC46 | . | . | . | . | . | . | . | . | . | . |
| CC49 | . | . | . | . | Asp | . | Phe | . | . | . |
| CC83 | . | . | . | . | Asp | . | . | . | . | . |
| CC92 | . | . | . | . | Asp | . | . | . | . | . |

CDR2 — 60

| $V_H$αTAG | Glu | Lys | Phe | Lys | Gly | Lys | Ala | Thr | Leu | Thr |
|---|---|---|---|---|---|---|---|---|---|---|
| CC46 | . | . | . | . | . | . | . | . | . | . |
| CC49 | . | Arg | . | . | . | . | . | . | . | . |
| CC83 | . | . | . | . | . | . | . | . | . | . |
| CC92 | . | . | . | . | . | . | . | . | . | . |

70

| $V_H$αTAG | Ala | Asp | Lys | Ser | Ser | Ser | Thr | Ala | Tyr | Met |
|---|---|---|---|---|---|---|---|---|---|---|
| CC46 | . | . | . | . | . | . | . | . | . | . |
| CC49 | . | . | . | . | . | . | . | . | . | . |
| CC83 | . | . | . | . | . | . | . | . | . | Val |
| CC92 | . | . | . | Pro | . | Asn | . | Val | . | . |

80

FIG. 3 (CONT.)

| $V_H\alpha$TAG | Gln | Leu | Asn | Ser | Leu | Thr | Ser | Glu | Asp | Ser |
|---|---|---|---|---|---|---|---|---|---|---|
| CC46 | . | Phe | . | . | . | . | . | . | . | . |
| CC49 | . | . | . | . | . | . | . | . | . | . |
| CC83 | . | . | . | . | . | . | . | . | . | . |
| CC92 | | | | | | | | | | |

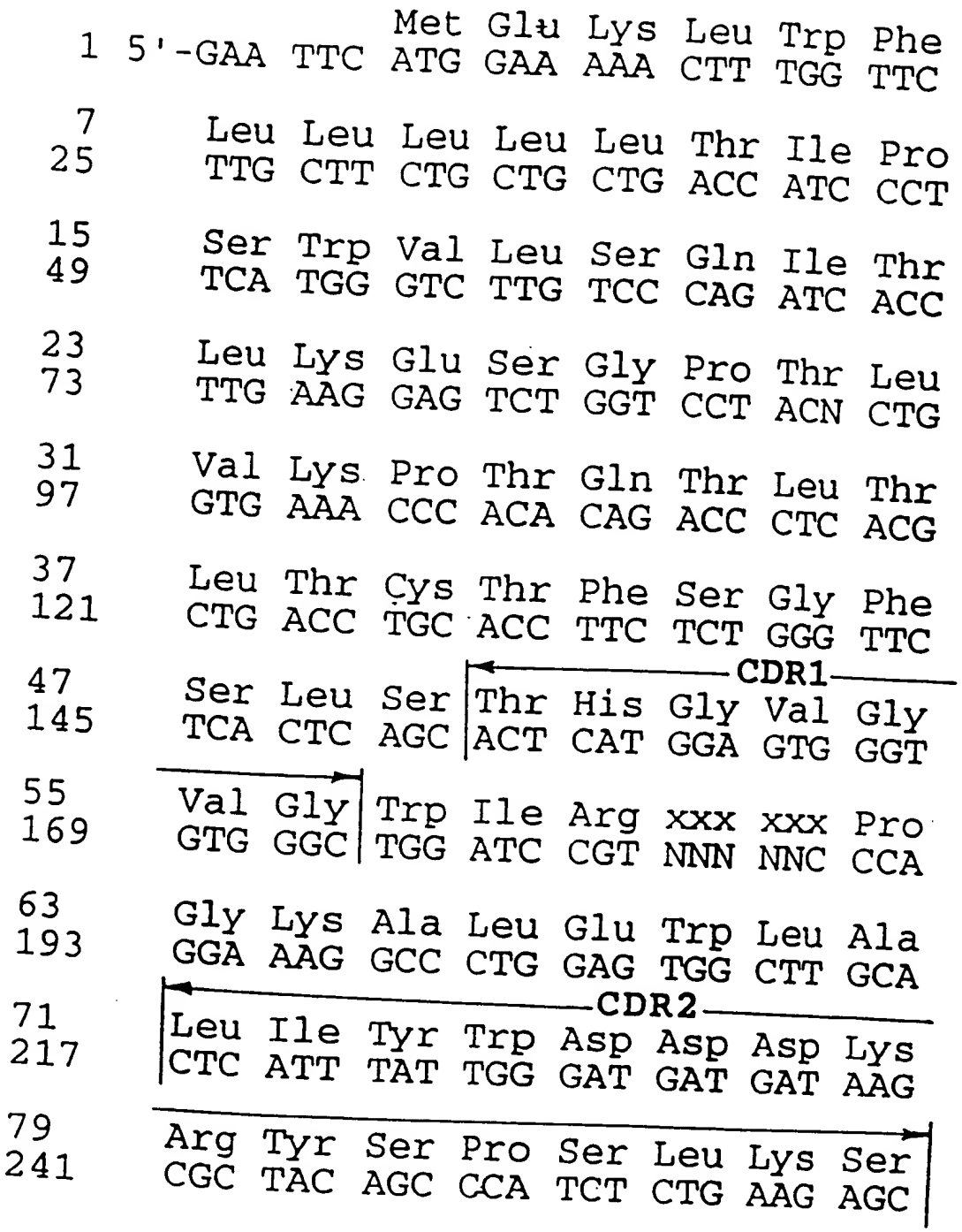
| $V_H\alpha$TAG | Ala | Val | 90 Tyr | Phe | Cys | Lys | Arg |
|---|---|---|---|---|---|---|---|
| CC46 | . | . | . | . | . | Thr | Gly |
| CC49 | . | . | . | . | . | Thr | . |
| CC83 | . | . | . | . | . | Arg | . |
| CC92 | . | . | . | . | . | Thr | . |

| | CDR3 | | | | | | | 105 |
|---|---|---|---|---|---|---|---|---|
| CC46 | Gly | Tyr | Gly | Val | Ala | Phe | Trp Gly | Gln |
| CC49 | Ser | Leu | Asn | Met | Ala | Tyr | Trp Gly | Gln |
| CC83 | Ser | Phe | Tyr | Gly | Asn | - | Trp Gly | Gln |
| CC92 | Ser | Leu | Ser | Gly | Asp | Ser | Trp Gly | Gln |

FIG. 3 (CONT.)

| | 110 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC46 | Gly | Thr | Leu | Val | Thr | Val | Ser | Ala |
| CC49 | Gly | Thr | Ser | Val | Thr | Val | Ser | Ser |
| CC83 | Gly | Thr | Thr | Leu | Thr | Val | Ser | Ser |
| CC92 | Gly | Thr | Thr | Leu | Thr | Val | Ser | Ser |

FIG. 4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7 Leu Leu Leu Leu Leu Thr Ile Pro
25 TTG CTT CTG CTG CTG ACC ATC CCT
15 Ser Trp Val Leu Ser Gln Ile Thr
49 TCA TGG GTC TTG TCC CAG ATC ACC
23 Leu Lys Glu Ser Gly Pro Thr Leu
73 TTG AAG GAG TCT GGT CCT ACN CTG
31 Val Lys Pro Thr Gln Thr Leu Thr
97 GTG AAA CCC ACA CAG ACC CTC ACG
37 Leu Thr Cys Thr Phe Ser Gly Phe
121 CTG ACC TGC ACC TTC TCT GGG TTC
CDR1
47 Ser Leu Ser Thr His Gly Val Gly
145 TCA CTC AGC ACT CAT GGA GTG GGT
55 Val Gly Trp Ile Arg xxx xxx Pro
169 GTG GGC TGG ATC CGT NNN NNC CCA
63 Gly Lys Ala Leu Glu Trp Leu Ala
193 GGA AAG GCC CTG GAG TGG CTT GCA
CDR2
71 Leu Ile Tyr Trp Asp Asp Asp Lys
217 CTC ATT TAT TGG GAT GAT GAT AAG
79 Arg Tyr Ser Pro Ser Leu Lys Ser
241 CGC TAC AGC CCA TCT CTG AAG AGC

FIG. 4 (CONT.)

```
87      Arg Leu Thr Ile Thr Lys Asp Thr
265     AGG CTC ACC ATC ACC AAG GAC ACC

95      Ser Lys Asn Gln Val Ile Leu Thr
289     TCC AAA AAC CAG GTG ATC CTT ACA

103     Met Thr Asn Met Asp Pro Val Asp
313     ATG ACC AAC ATG GAC CCT GTG GAC

111     Thr Ala Thr Tyr Tyr Cys Ala His
337     ACA GCC ACA TAT TAT TGT GCA CAC
        <------------ CDR3 ------------>
119     Gly Leu Pro Ser Met Val Lys Asn
361     GGG CTG CCA TCT ATG GTT AAG AAC

127     Trp Gly Gln Gly Thr Thr Val Thr
385     TGG GGC CAA GGG ACC ACG GTC ACC

135     Val Ser Ser Gly Ser
409     GTC TCC TCA GGG AGT-3'
```

FIG. 5

MOUSE GERMLINE J-H GENES FROM pNP9

5'-GGATCCTGGC CAGCATTGCC GCTAGGTCCC
TCTCTTCTAT GCTTTCTTTG TCCCTCACTG
GCCTCCATCT GAGATAATCC TGGAGCCCTA
GCCAAGGATC ATTTATTGTC AGGGGTCTAA
TCATTGTTGT CACAATGTGC CTGGTTTGCT
TACTGGGGCC AAGGGACTCT GGTCACTGTC
TCTGCAGGTG AGTCCTAACT TCTCCCATTC
TAAATGCATG TTGGGGGGAT TCTGAGCCTT
CAGGACCAAG ATTCTCTGCA AACGGGAATC
AAGATTCAAC CCCTTTGTCC CAAAGTTGAG
ACATGGGTCT GGGTCAGGGA CTCTCTGCCT
GCTGGTCTGT GGTGACATTA GAACTGAAGT
ATGATGAAGG ATCTGCCAGA ACTGAAGCTT
GAAGTCTGAG GCAGAATCTT GTCCAGGGTC
TATCGGACTC TTGTGAGAAT TAGGGGCTGA
CAGTTGATGG TGACAATTTC AGGGTCAGTG
ACTGTCAGGT TTCTCTGAGG TGAGGCTGGA
ATATAGGTCA CCTTGAAGAC TAAAGAGGGG
TCCAGGGGCT TTTCTGCACA GGCAGGGAAC
AGAATGTGGA ACAATGACTT GAATGGTTGA
TTCTTGTGTG ACACCAAGAA TTGGCATAAT
GTCTGAGTTG CCCAAGGGTG ATCTTAGCTA
AAAACCCACT ATTGTGATTA CTATGCTATG
GACTACTGGG GTCAAGGAAC CTCAGTCACC
GTCTCCTCAG GTAAGAATGG CCTCTCCAGG
TCTTTATTTT TAACCTTTGT TATGGAGTTT
TCTGAGCATT GCAGACTAAT CTTGGATATT
TGCCCTGAGG GAGCCGGCTG AGAGAAGTTG
GGAAATAAAT CTGTCTAGGG ATCTCAGAGC
CTTTAGGACA GATTATCTCC ACATCTTTGA
AAAACTAAGA ATCTGTGTGA TGGTGTTGGT
GGAGTCCCTG GATGATGGGA TAGGGACTTT

# FIG. 5 (CONT.)

GGAGGCTCAT TTGAGGGAGA TGCTAAAACA
ATCCTATGGC TGGAGGGATA GTTGGGGCTG
TAGTTGGAGA TTTTCAGTTT TTAGAATGAA
GTATTAGCTG CAATACTTCA AGGACCACCT
CTGTGACAAC CATTTTATAC AGTATCCAGG
CATAGGGACA AAAAGTGGAG TGGGGCACTT
TCTTTAGATT TGTGAGGAAT GTTCCACACT
AGATTGTTTA AAACTTCATT TGTTGGAAGG
AGCTGTCTTA GTGATTGAGT CAAGGGAGAA
AGGCATCTAG CCTCGGTCTC AAAAGGGTAG
TTGCTGTCTA GAGAGGTCTG GTGGAGCCTG
CAAAAGTCCA GCTTTCAAAG GAACACAGAA
GTATGTGTAT GGAATATTAG AAGATGTTGC
TTTTACTCTT AAGTTGGTTC CTAGGAAAAA
TAGTTAAATA CTGTGACTTT AAAATGTGAG
AGGGTTTTCA AGTACTCATT TTTTTAAATG
TCCAAAATTT TTGTCAATCA ATTTGAGGTC
TTGTTTGTGT AGAACTGACA TTACTTAAAG
TTTAACCGAG GAATGGGAGT GAGGCTCTCT
CATACCCTAT TCAGAACTGA CTTTTAACAA
TAATAAATTA AGTTTAAAAT ATTTTTAAAT
GAATTGAGCA ATGTTGAGTT GAGTCAAGAT
GGCCGATCAG AACCGGAACA CCTGCAGCAG
CTGGCAGGAA GCAGGTCATG TGGCAAGGCT
ATTTGGGGAA GGGAAAATAA AACCACTAGG
TAAACTTGTA GCTGTGGTTT GAAGAAGTGG
TTTTGAAACA CTCTGTCCAG CCCCACCAAA
CCGAAAGTCC AGGCTGAGCA AAACACCACC
TGGGTAATTT GCATTTCTAA AATAAGTTGA
GGATTCAGCC GAAACTGGAG AGGTCCTCTT
TTAACTTATT GAGTTCAACC TTTTAATTTT
AGCTTGAGTA GTTCTAGTTT CCCCAAACTT
AAGTTTATCG ACTTCTAAAA TGTATTTAGA
ATTC-3'

Fig. 6

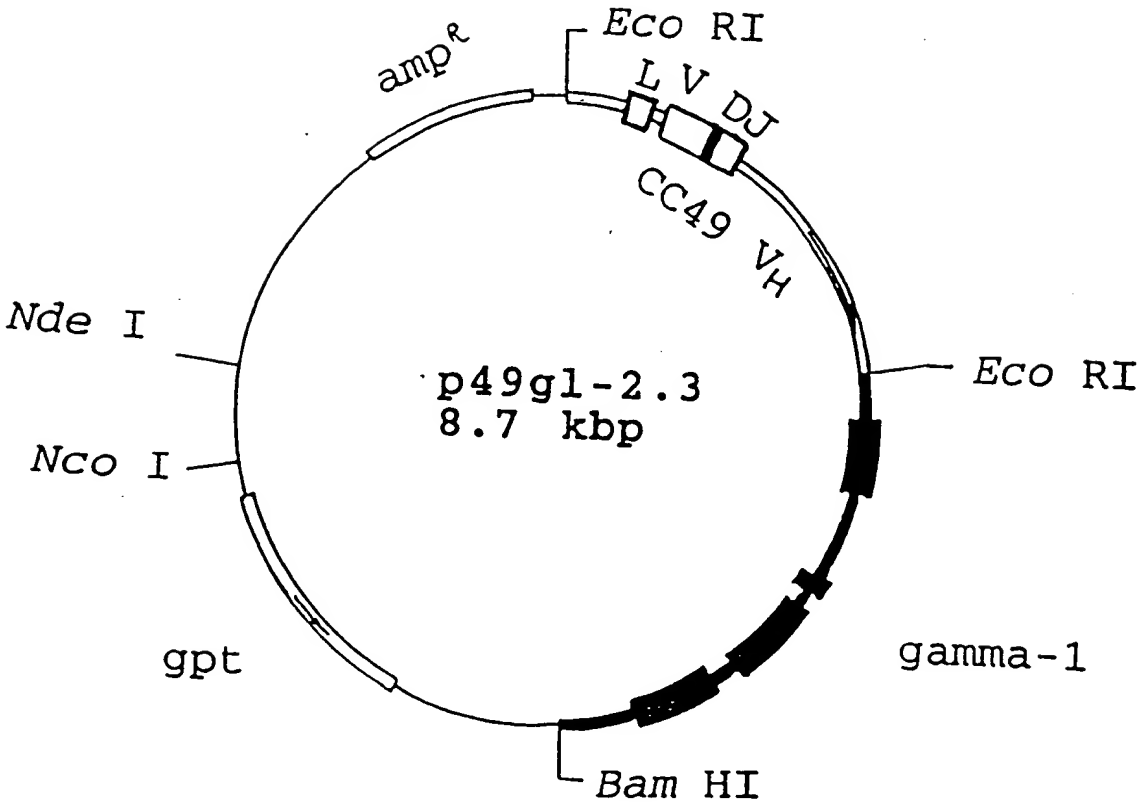

amp$^R$
Eco RI
L V DJ
CC49 VH
Nde I
Nco I
p49g1-2.3
8.7 kbp
Eco RI
gpt
gamma-1
Bam HI

FIG. 7

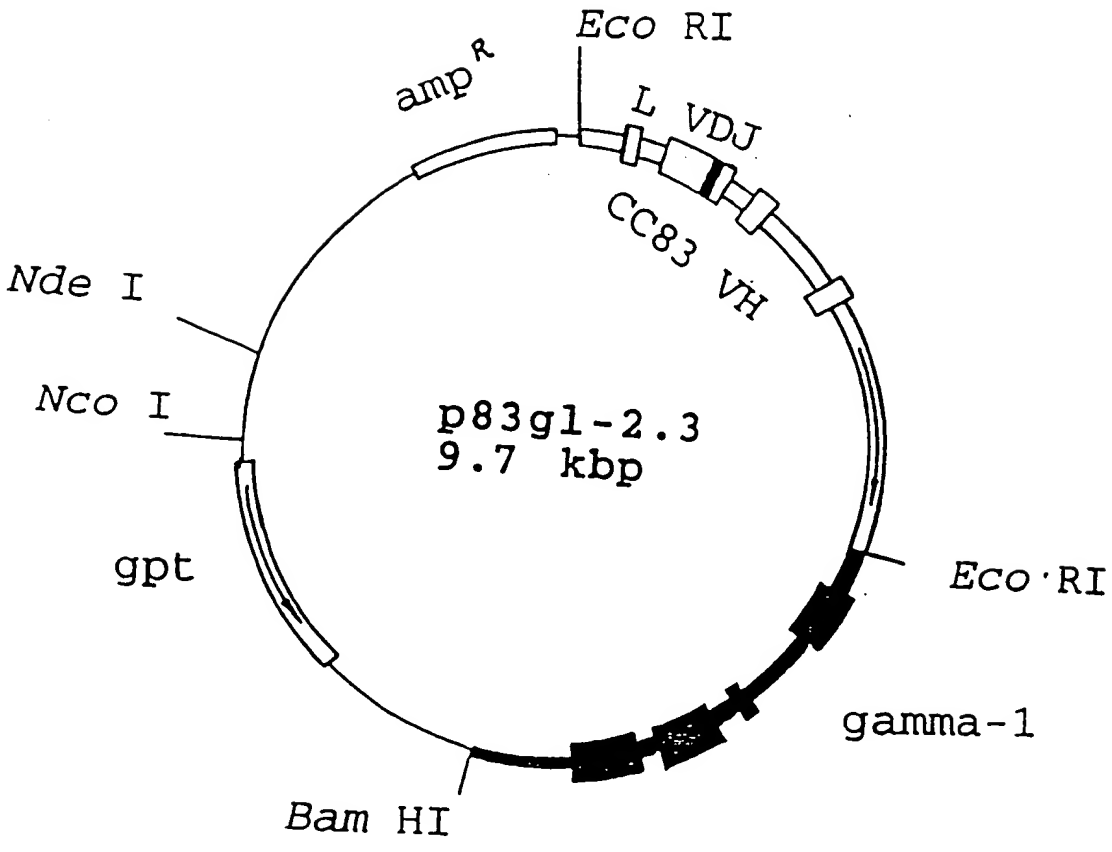

Eco RI
amp R
L
VDJ
CC83 VH
Nde I
Nco I
p83g1-2.3
9.7 kbp
gpt
Eco RI
gamma-1
Bam HI

FIG. 8

HUMVL (+), 26-MER:
(*Cla* I)
5'- GAAGAG**TATC GAT**AAAATTT ATTGAG-3'

HUMVL(-), 98-MER:

(SPLICE SITE)
(*Hind* III)
5'-CATT**AAGCTT** AGAAAAGTGT ACTTACGTTT
GATCACCACC TTGGTCCCTC CGCCGAAAGT
GAGAGGATAA CTATAATATT GCTGACAGTA
ATAAACTG-3'

FIG. 9

HJ4:

Leu Thr Phe Gly Gly Gly Thr Lys
CTC ACT TTC GGC GGA GGG ACC AAG

Val Glu Ile Lys A(rg)
GTG GAG ATC AAA C GTAAGTGCAC

TTTCCTAA

FIGURE 9: Human J4 (HJ4) amino acid and DNA sequences. The first two amino acids (Leu-Thr) complete the CDR3 region, the remainder make up the FR4 region. The (↓) indicates the splice site and the beginning of the intron between the J and C exons. DNA sequence underlined in HJ4 represents a part of the sequence used for the 3' end PCR oligo HUMVL(-).

FIG. 10


Cla I
5' ATCGATAAAA TTTATTGAGA ATTTGTTTAT TATGATTAAC 3418
3' TAGCTATTTT AAATAACTCT TAAACAAATA ATACTAATTG
AGAGGTAAAA GCCAGTATAT TACTGATTAA TATAGGTAAA 3458
TCTCCATTTT CGGTCATATA ATGACTAATT ATATCCATTT
*
AGGCAGTTAA GAAATTGGGA ATGCTTTCTC TTCTGCTTTC 3498
TCCGTCAATT CTTTAACCCT TACGAAAGAG AAGACGAAAG
TTCTACGATG CACAAGGCGT TTCACATTTA TGCCCCTATG 3538
AAGATGCTAC GTGTTCCGCA AAGTGTAAAT ACGGGGATAC
AAAATTACTA GGCTGTCCTA GTCATTAGAT CTTTCAGCAG 3578
TTTTAATGAT CCGACAGGAT CAGTAATCTA GAAAGTCGTC
TTTGTAGTTT TAGAGCTTCT AAGTTGACTT CTGTCTTTTC 3618
AAACATCAAA ATCTCGAAGA TTCAACTGAA GACAGAAAAG
TATTCATACA ATTACACATT CTGTGATGAT ATTTTTGGCT 3658
ATAAGTATGT TAATGTGTAA GACACTACTA TAAAAACCGA
HUMLIN1 (-)

FIG. 10 (CONT.)

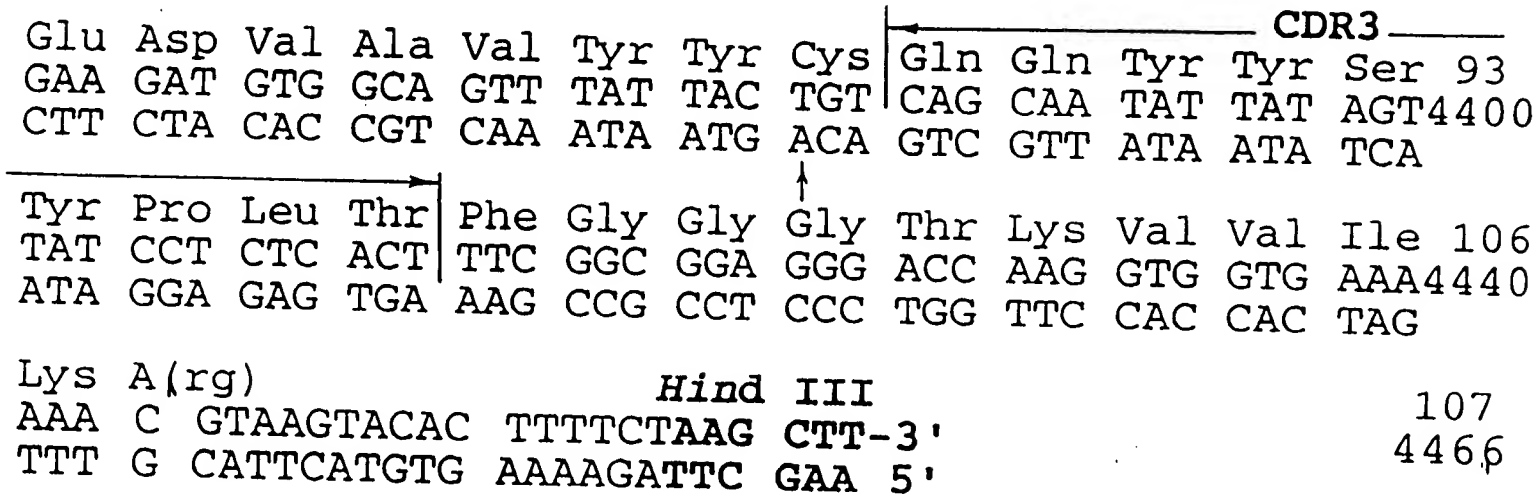
```
AAAAAAATTA AGATATAGTT GGAAATAATG ACTATTTCCA
TTTTTTTAAT TCTATATCAA CCTTTATTAC TGATAAAGGT
     Bam HI
ATATGGATCC AATTATCTGC TGACTTATAA TACTACTAGA
TATACCTAGG TTAATAGACG ACTGAATATT ATGATGATCT

AAGCAAATTT AAATGACATA TTTCAATTAT ATCTGAGACA
TTCGTTTAAA TTTACTGTAT AAAGTTAATA TAGACTCTGT

GCGTGTATAA GTTTATGTAT AATCATTGTC CATTACTGAC
CGCACATATT CAAATACATA TTAGTAACAG GTAATGACTG

TACAG
ATGTC
```

```
                    +1
ly Ala Tyr Gly Asp Ile Val Met Thr Gln Ser          7
GT GCC TAC GGG GAC ATC GTG ATG ACC CAG TCT       4125
CA CGG ATG CCC CTG TAG CAC TAC TGG GTC AGA
```

FIG. 10 (CONT.)

```
Pro Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Ala Thr 20
CCA GAC TCC CTG GCT GTG TCT CTG GGC GAG AGG GCC ACC4164
GGT CTG AGG GAC CGA CAC AGA GAC CCG CTC TCC CGG TGG

                    <-------------CDR1-------------
Ile Asn Cys Lys Ser Ser Gln Ser Val Leu Tyr Ser Ser 27F
ATC AAC TGC AAG TCC AGC CAG AGT GTT TTA TAC AGC TCC4203
TAG TTG ACG TTC AGG TCG GTC TCA CAA AAT ATG TCG AGG

-------------------->
Asn Asn Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro 40
AAC AAT AAG AAC TAC TTA GCT TGG TAC CAG CAG AAA CCA4242
TTG TTA TTC TTG ATG AAT CGA ACC ATG GTC GTC TTT GGT
      HUMLCDR1(-)
                                    <-------CDR2-----
Gly Gln Pro Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr 53
GGA CAG CCT CCT AAG CTG CTC ATT TAC TGG GCA TCT ACC4281
CCT GTC GGA GGA TTC GAC GAG TAA ATG ACC CGT AGA TGG

------------->
Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly 66
CGG GAA TCC GGG GTC CCT GAC CGA TTC AGT CGC AGT CGC4320
GCC CTT AGG CCC CAG GGA CTG GCT AAG TCA CCG TCG CCC

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGC CTG CAG GCT
AGA CCC TGT CTA AAG TGA GAG TGG TAG TCG TCG GAC GTC CGA
```

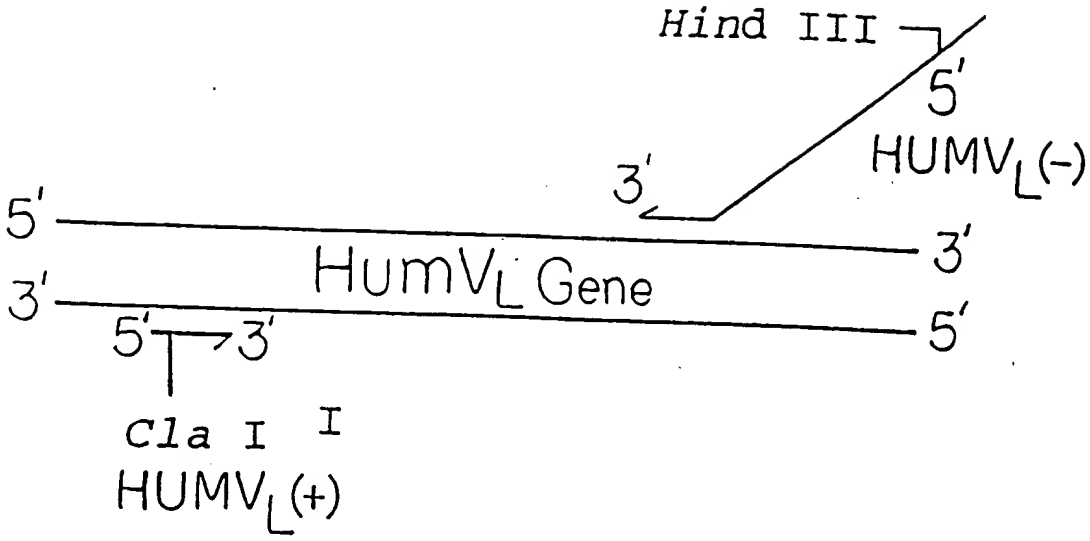
FIG. 10 (CONT.)


CDR3
Glu Asp Val Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser 93
GAA GAT GTG GCA GTT TAT TAC TGT CAG CAA TAT TAT AGT4400
CTT CTA CAC CGT CAA ATA ATG ACA GTC GTT ATA ATA TCA
Tyr Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Val Ile 106
TAT CCT CTC ACT TTC GGC GGA GGG ACC AAG GTG GTG AAA4440
ATA GGA GAG TGA AAG CCG CCT CCC TGG TTC CAC CAC TAG
Lys A(rg)
Hind III
AAA C GTAAGTACAC TTTTCTAAG CTT-3'
TTT G CATTCATGTG AAAAGATTC GAA 5'
107
4466

# FIG. 10 (CONT.)

FIGURE 10: Entire DNA sequence of the Hum4 V gene *Cla* I-*Hind* III segment in pRL1001, Clone #2. A single base difference occured at position 3461 and is marked with an asterisk (*). The corresponding amino acid sequences in the coding exons are shown. The site of the Leu-Pro mutation in Clone #7 is boxed. An arrow () indicates the site of the single base deletion in Clone #11. Oligonucleotides used as primers for the sequencing reactions are underlined. In order as they occur from the 5' end are: HUMLIN1(-), HUMLIN2(-), HUMLCDR1(-) and *Hind* III Ck(-)(not shown).

FIG. 11




A schematic representation of the human germline Subgroup IV gene (HumV$_L$) as the target for the PCR. The 5'-end oligo (HUMVL(+)) and the 3'end oligo (HUMVL(-)) used to prime the elongation reactions for Taq polymerase are shown with half-arrows to indicate the direction of synthesis.

FIG. 12

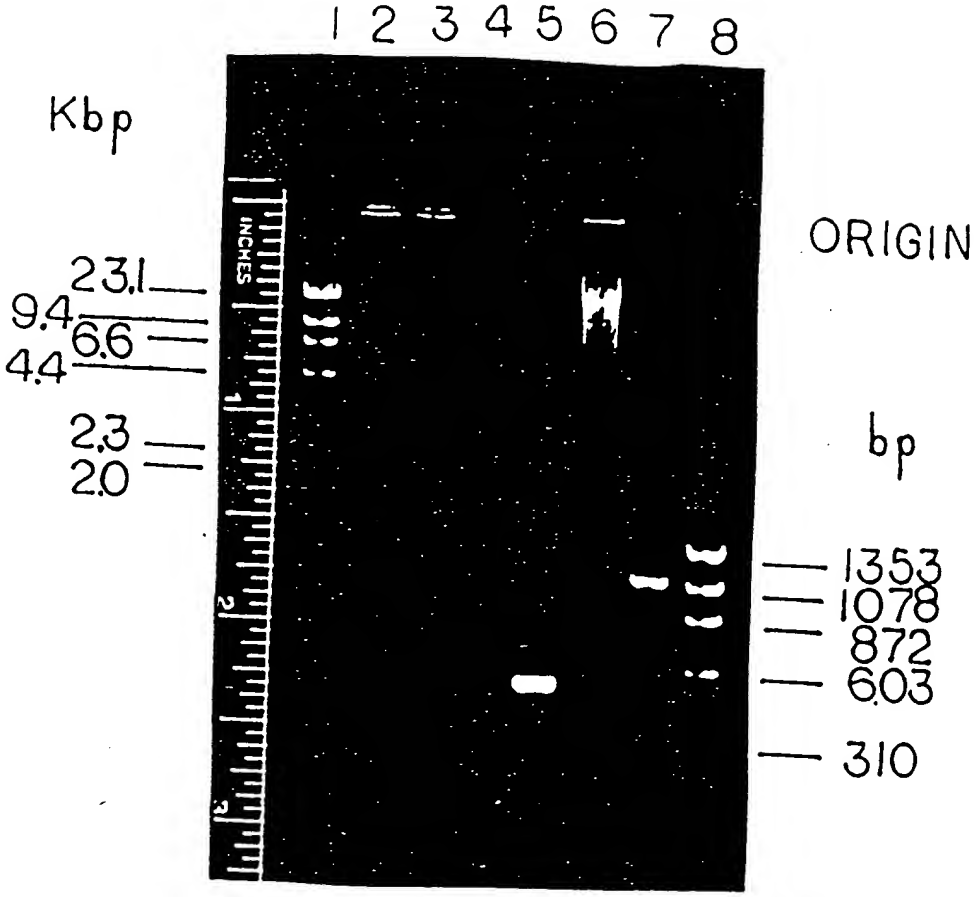



FIGURE 12: Agarose gel electrophoresis of Hum4V$_L$ PCR reactions. Lane 1: λ *Hind* III standard; lane 2: no Taq polymerase control; lane 3: no primers added; lane 4: no human DNA template; lane 5: Gene *Amp* kit positive control; lane 6: 3 μg human DNA with primers and Taq polymerase; lane 7: same as lane 6, but with 1ug human DNA and lane 8: ØX174-*Hae* III DNA standard. Ethidium bromide was added to the gel and buffer. Bands were visualized by long wavelength UV light.

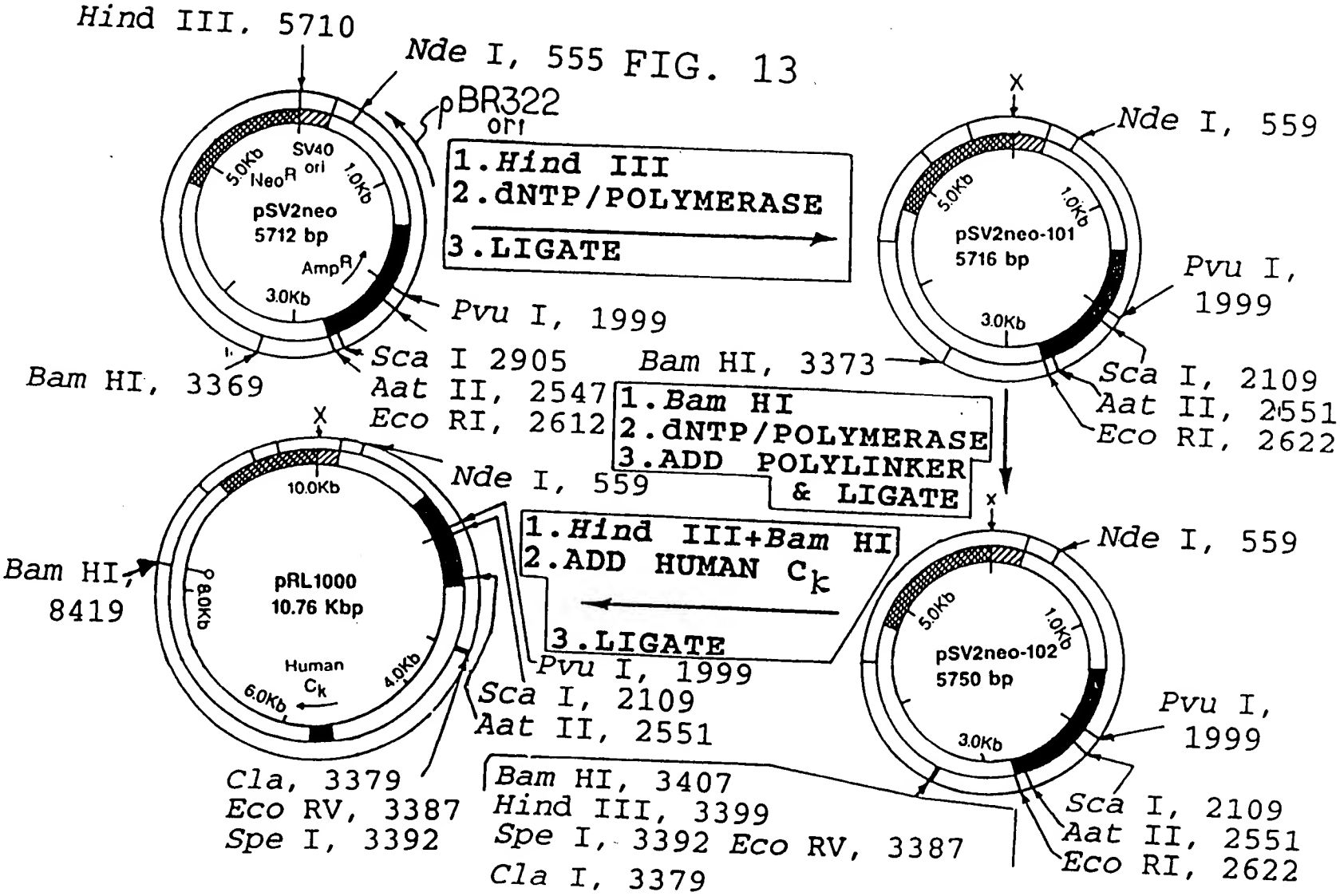

Hind III, 5710
Nde I, 555
pBR322 ori
SV40 ori
NeoR
5.0Kb
1.0Kb
pSV2neo
5712 bp
AmpR
3.0Kb
1. Hind III
2. dNTP/POLYMERASE
3. LIGATE
Pvu I, 1999
Sca I 2905
Aat II, 2547
Eco RI, 2612
Bam HI, 3369
X
Nde I, 559
pSV2neo-101
5716 bp
Pvu I, 1999
Bam HI, 3373
Sca I, 2109
Aat II, 2551
Eco RI, 2622
1. Bam HI
2. dNTP/POLYMERASE
3. ADD POLYLINKER & LIGATE
10.0Kb
pRL1000
10.76 Kbp
8.0Kb
Human Ck
4.0Kb
6.0Kb
Bam HI, 8419
Nde I, 559
1. Hind III+Bam HI
2. ADD HUMAN Ck
3. LIGATE
Pvu I, 1999
Sca I, 2109
Aat II, 2551
Cla, 3379
Eco RV, 3387
Spe I, 3392
pSV2neo-102
5750 bp
Nde I, 559
Pvu I, 1999
Sca I, 2109
Aat II, 2551
Eco RI, 2622
Bam HI, 3407
Hind III, 3399
Spe I, 3392
Eco RV, 3387
Cla I, 3379

FIG. 14

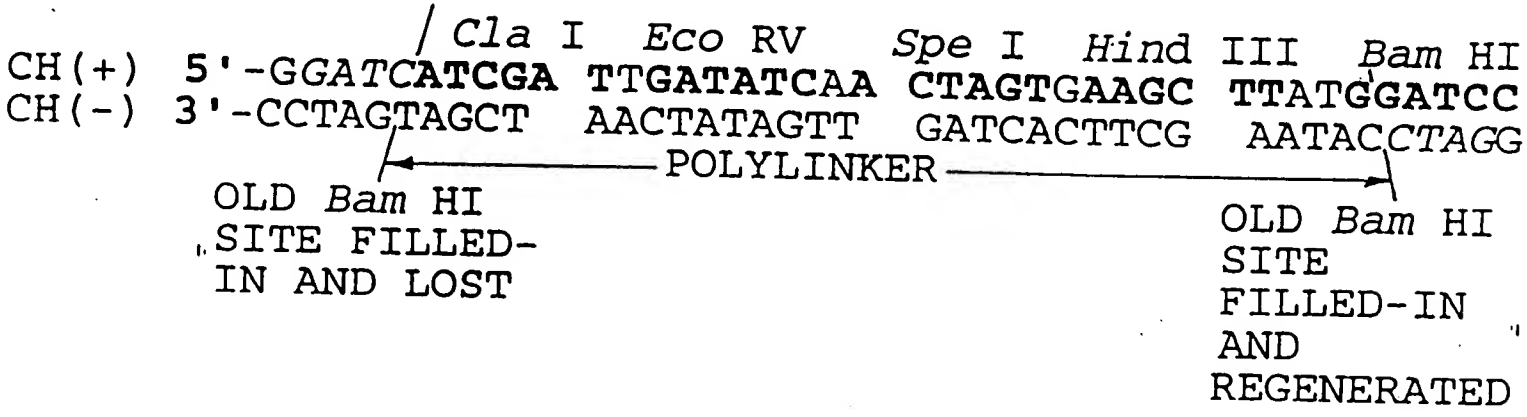



FIGURE 14: The polylinker inserted between filled-in *Bam* HI site of pSV2neo-101 to create pSV2neo-102. Note that the polylinker could be inserted in both orientations such that the *Bam* HI site on the left side could also be regenerated (and the one on the right side lost). The nucleotides used to fill-in the *Bam* HI site are shown in italics. The top synthetic oligo was called CH(+) while the complimentary strand was CH(-).

# FIG. 15

(A portion of the DNA Sequence of pSV2neo)

←——TOWARDS *Eco* RI SITE 5'-GAGGAGGTTA

GGGTTTATGA GGACACAGAG GAGCTTCCTG

GGGATCCAGA CATGATAAGA TACATTGATG

*Bam* H1

AGTTTGGACA AACCACAACT AGA-3'

FIGURE 15: Oligonucleotide synthesized (21-mer, called NEO102SEQ) to sequence putative pSV2neo-102 clones is the underlined sequence shown above. The *Bam* HI site where the polylinker was inserted in pSV2neo-101 is boxed.

FIG. 16

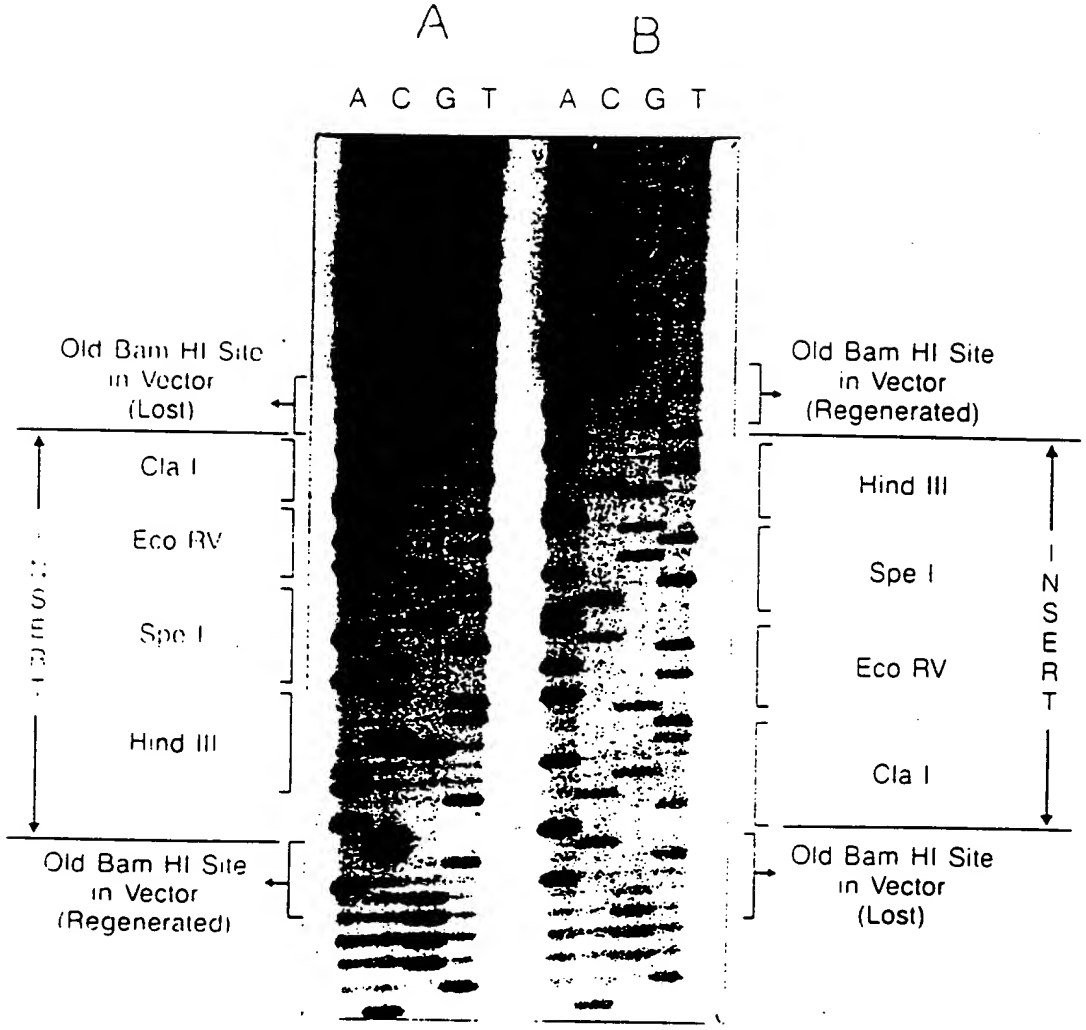



FIGURE 16: P-32 autoradiogram showing polylinker DNA sequence cloned in *Bam* HI site of pSV2neo-101. In both cases a single 30-base linker unit was incorporated, but in opposite orientations. Panel A-Sequence resulting in pSV2neo-120; Panel B-sequence resulting in pSV2neo-102. Reading the sequence (going up) is in the 5' to 3' direction of the (+) strand.

FIG. 17

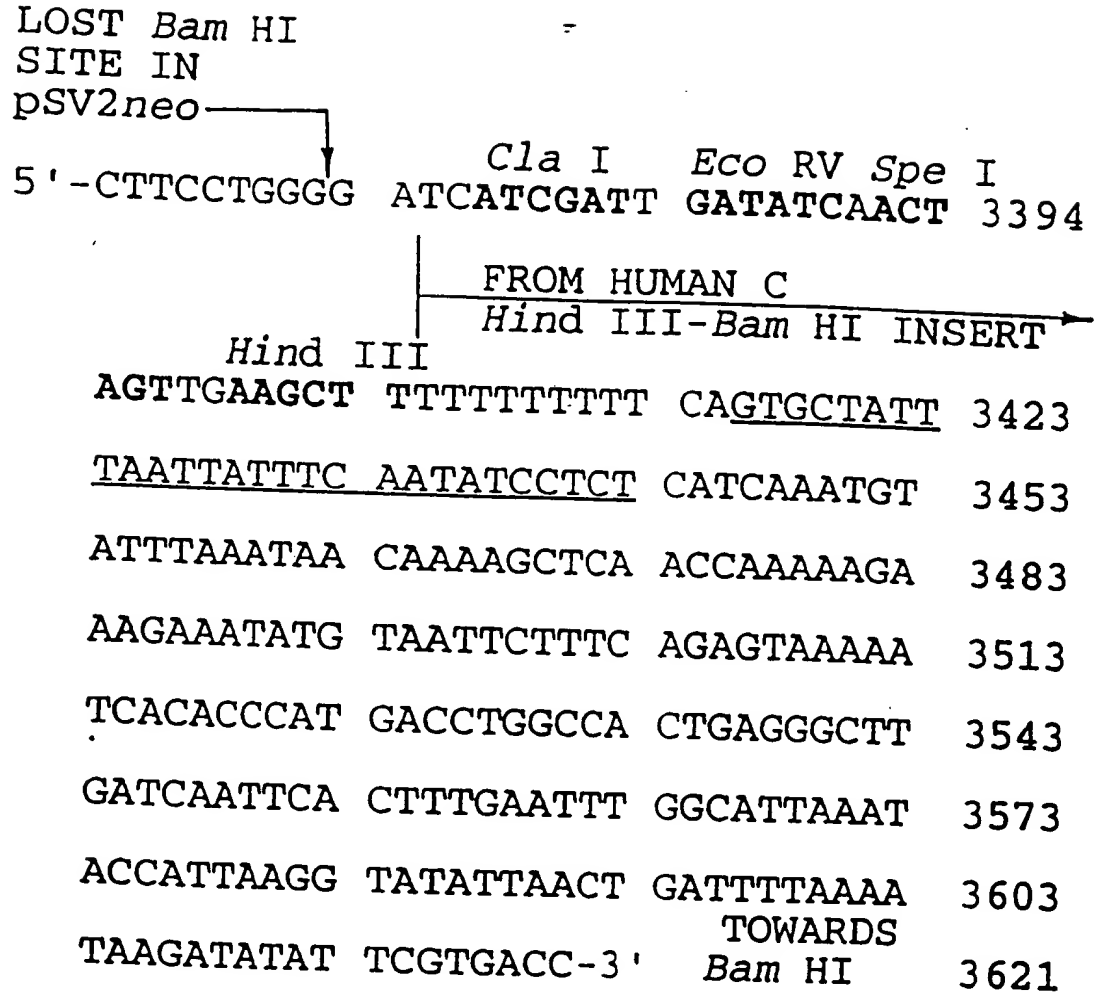



FIGURE 17: DNA sequence from pRL1000, reading the (+) strand from the primer NEO102SEQ (Figure 15). Sequence data past the *Hind* III site is from the human $C_K$ Hind III - *Bam* HI insert. The sequence complementary to the underlined DNA sequence, called *Hind* III$C_K$(-), was synthesized as a primer for sequencing in the upstream 3' direction.

FIG. 18

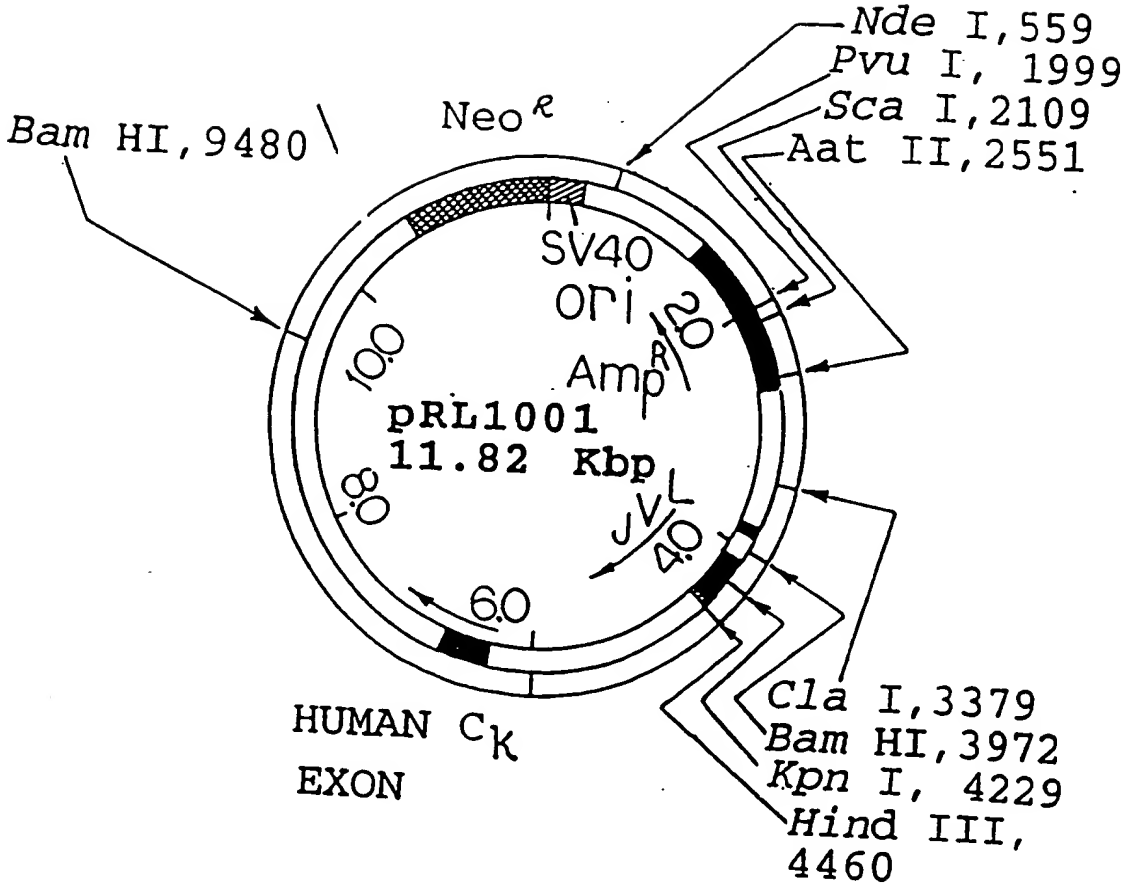



FIGURE 18: Partial restriction map of the plasmid pRL1001. This is the expression vector to introduce the human anti-tumor L chain gene in Sp2/0 cells.

FIG. 19

DNA SEQUENCING - pRL1001

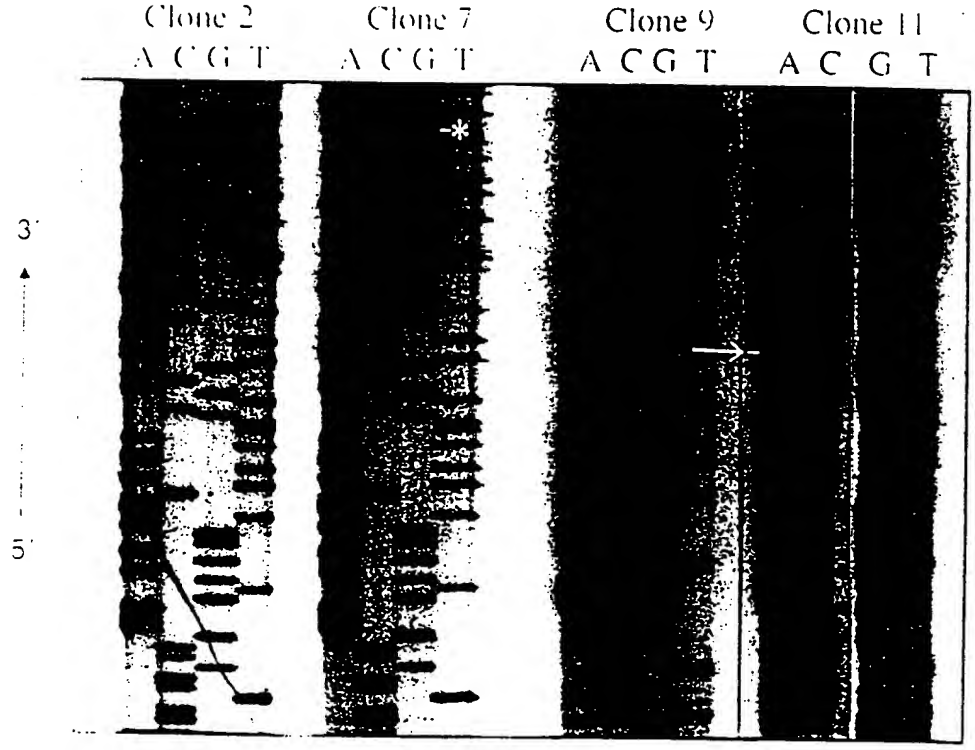
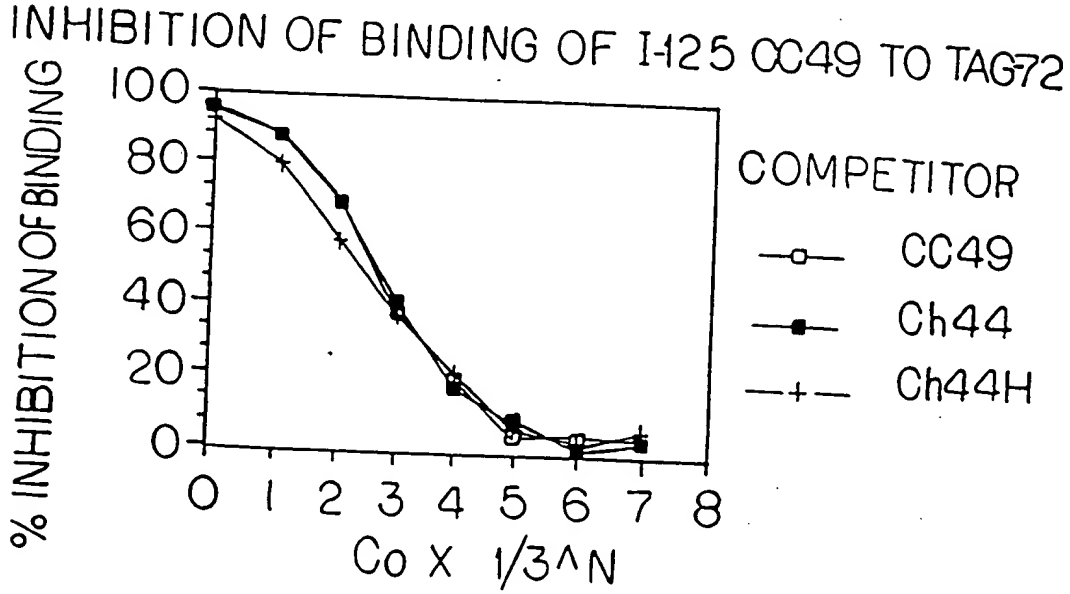



FIGURE 19: DNA sequence autoradiogram of pRL1001 clones. Reading the gel is in the 5' to 3' direction on the (-) strand, from the *Hind* III $C_{\kappa}$(-) primer. Clones 2 and 9 were equivalent to the expected sequence, clone 7 had a single base substitution (marked by *) and clone 11 had a single base deletion (marked by -)

Fig. 20

RECIPROCAL COMPETITION BETWEEN CC49, Ch44, AND Ch44H



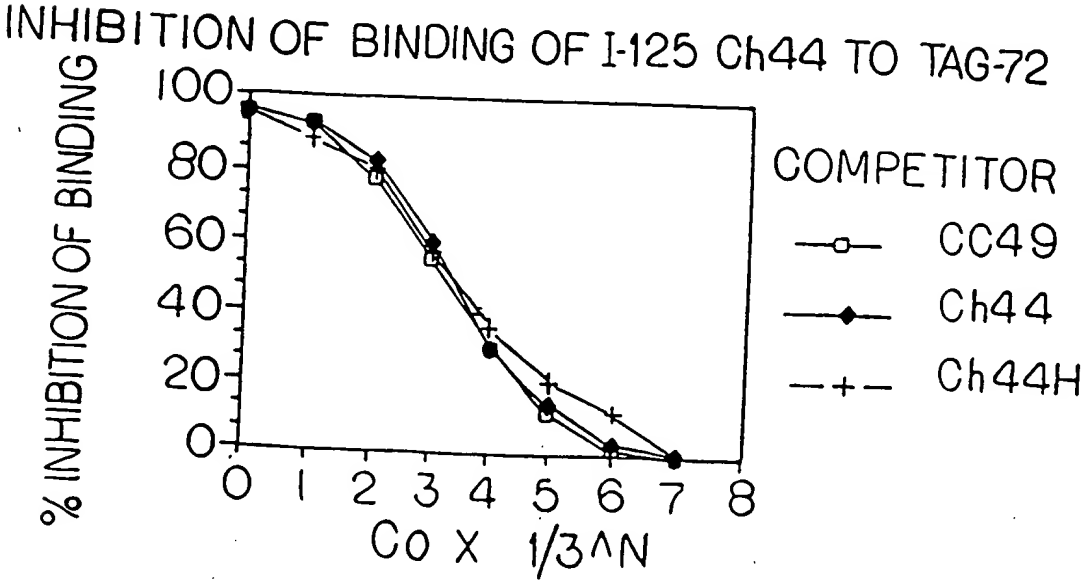



Fig. 20 (CONT.)

RECIPROCAL COMPETITION BETWEEN

CC49, Ch44, AND Ch44H

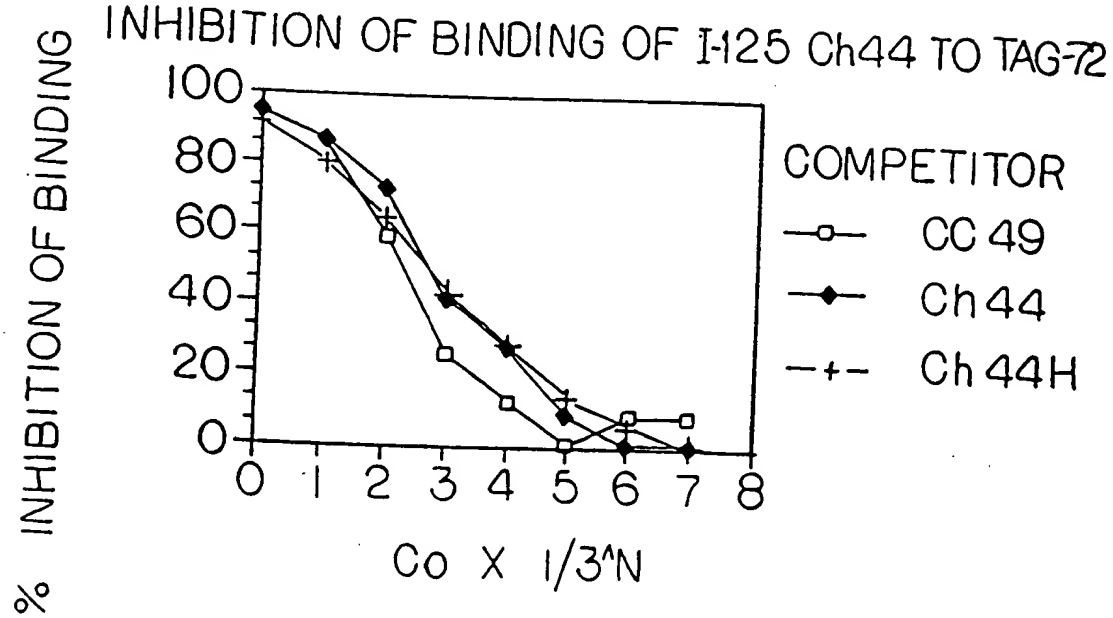


FIG. 21

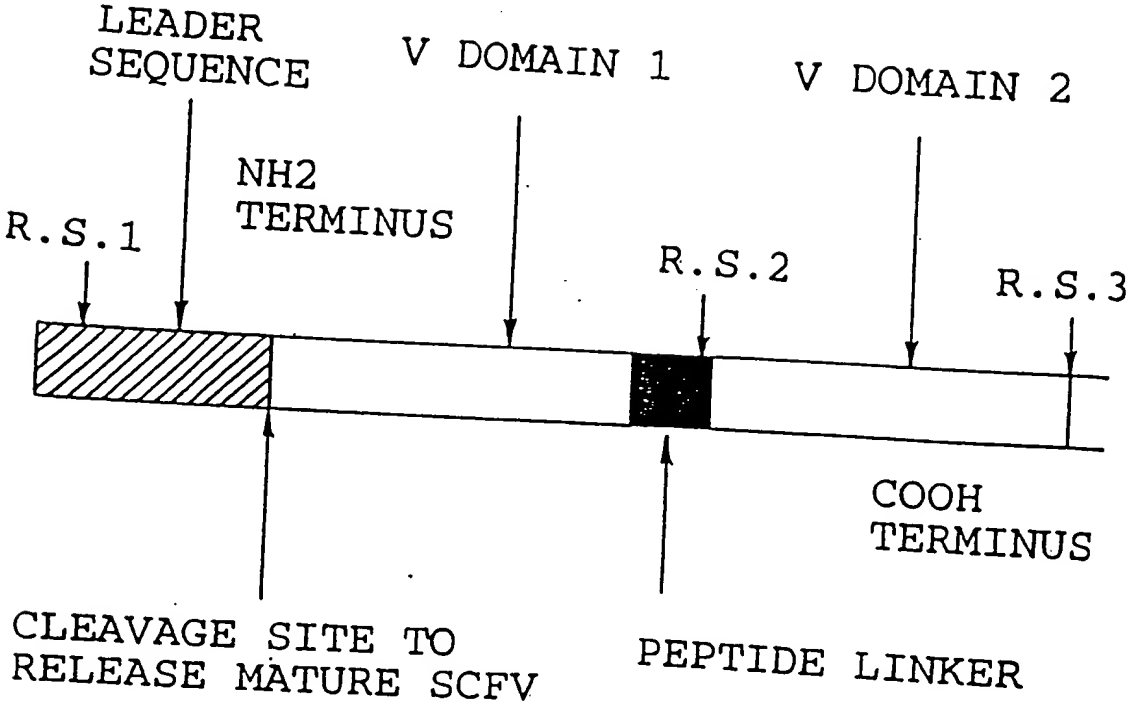

LEADER SEQUENCE
V DOMAIN 1
V DOMAIN 2
NH2 TERMINUS
R.S.1
R.S.2
R.S.3
COOH TERMINUS
CLEAVAGE SITE TO RELEASE MATURE SCFV
PEPTIDE LINKER

FIG. 22 (CONT.)

Gly Thr Asp Phe Thr Leu Thr Ile Ser
GGG ACA GAT TTC ACT CTC ACC ATC AGC

Ser Leu Gln Ala Glu Asp Val Ala Val
AGC CTG CAG GCT GAA GAT GTG GCA GTT

Tyr Tyr Cys | Gln Gln Tyr Tyr Ser Tyr
TAT TAC TGT | CAG CAA TAT TAT AGT TAT

Pro Leu Thr | Phe Gly Gly Gly Thr Lys
CCT CTC ACT | TTC GGC GGA GGG ACC AAG

Val | Lys Glu Ser Gly Ser Val Ser Ser
GTG | AAG GAG TCA GGT TCG GTC TCC TCA

Glu Gln Leu Ala Gln Phe Arg Ser Leu
GAA CAA TTG GCC CAA TTT CGT TCC TTA

Asp | Val Gln Leu Gln Gln Ser Asp Ala
GAC | GTC CAG TTG CAG CAG TCT GAC GCT

Glu Leu Val Lys Pro Gly Ala Ser Val
GAG TTG GTG AAA CCT GGG GCT TCA GTG

Lys Ile Ser Cys Lys Ala Ser Gly Tyr
AAG ATT TCC TGC AAG GCT TCT GGC TAC

Thr Phe Thr | Asp His Ala Ile His | Trp
ACC TTC ACT | GAC CAT GCA ATT CAC | TGG

Val Lys Gln Asn Pro Glu Gln Gly Leu
GTG AAA CAG AAC CCT GAA CAG GGC CTG

CDR3L

LINKER

CDR1H

FIG. 22 (CONT.)

```
Glu Trp Ile Gly | Tyr Phe Ser Pro Gly
GAA TGG ATT GGA | TAT TTT TCT CCC GGA
                CDR2H
Asn Asp Asp Phe Lys Tyr Asn Glu Arg
AAT GAT GAT TTT AAA TAC AAT GAG AGG

Phe Lys Gly | Lys Ala Thr Leu Thr Ala
TTC AAG GGC | AAG GCC ACA CTG ACT GCA

Asp Lys Ser Ser Ser Thr Ala Tyr Val
GAC AAA TCC TCC AGC ACT GCC TAC GTG

Gln Leu Asn Ser Leu Thr Ser Glu Asp
CAG CTC AAC AGC CTG ACA TCT GAG GAT

Ser Ala Val Tyr Phe Cys Thr Arg | Ser
TCT GCA GTG TAT TTC TGT ACA AGA | TCC
CDR3H
Leu Asn Met Ala Tyr | Trp Gly Gln Gly
CTG AAT ATG GCC TAC | TGG GGT CAA GGA

Thr Ser Val Thr Val Ser
ACC TCA GTC ACC GTC TCC TAG TGA

AGCTTGGAAC ACCACACAAA CCATATCCAA A
```

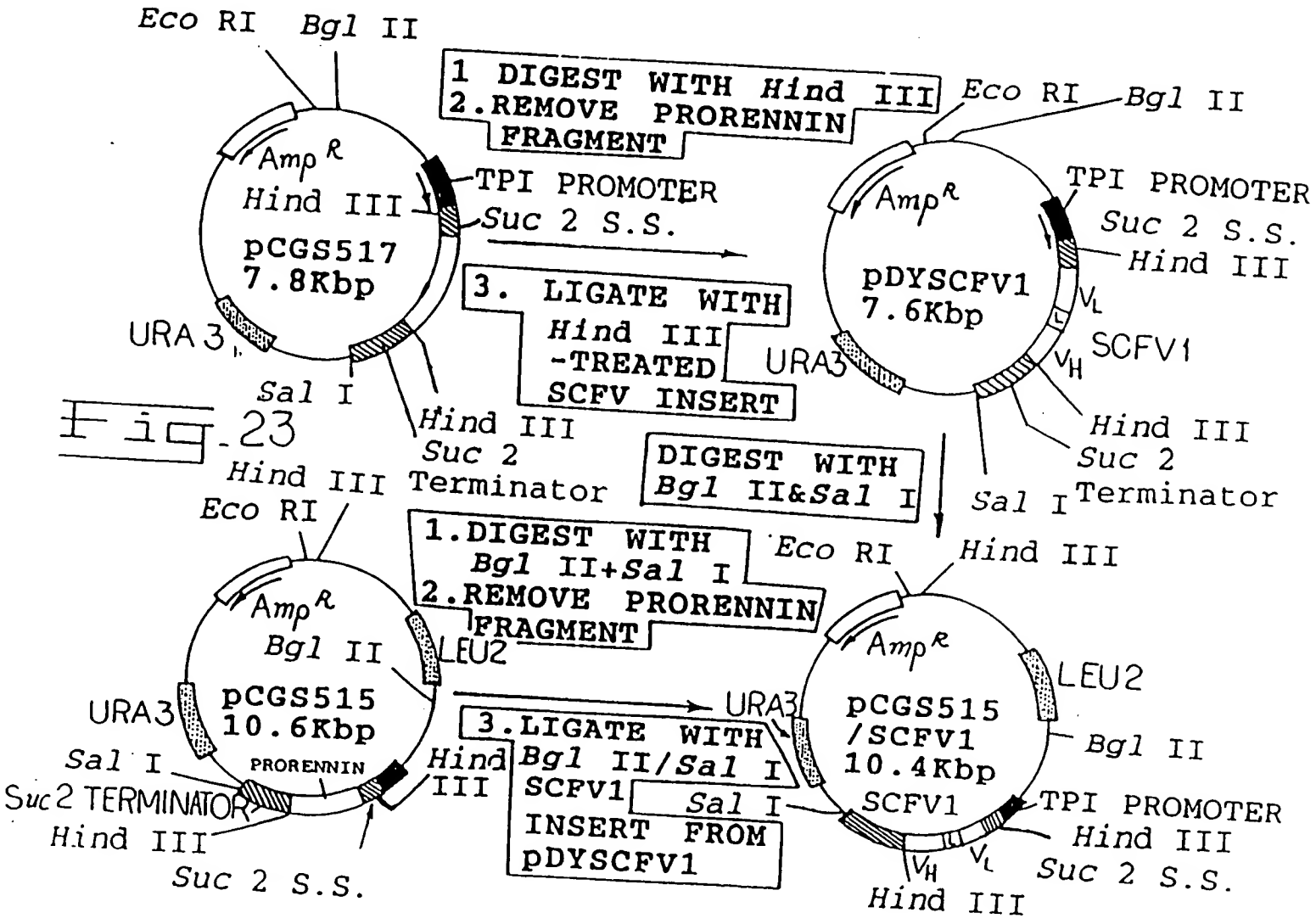

Eco RI
Bgl II
1 DIGEST WITH Hind III
2.REMOVE PRORENNIN FRAGMENT
Amp R
Hind III
pCGS517
7.8Kbp
TPI PROMOTER
Suc 2 S.S.
URA 3
Sal I
Hind III
Suc 2 Terminator
3. LIGATE WITH Hind III -TREATED SCFV INSERT
Eco RI
Bgl II
Amp R
pDYSCFV1
7.6Kbp
TPI PROMOTER
Suc 2 S.S.
Hind III
VL
SCFV1
VH
URA3
Hind III
Suc 2 Terminator
Sal I
DIGEST WITH Bgl II&Sal I
Fig. 23
Hind III
Eco RI
Amp R
Bgl II
pCGS515
10.6Kbp
PRORENNIN
LEU2
URA3
Sal I
Suc2 TERMINATOR
Hind III
Suc 2 S.S.
1.DIGEST WITH Bgl II+Sal I
2.REMOVE PRORENNIN FRAGMENT
3.LIGATE WITH Bgl II/Sal I SCFV1 INSERT FROM pDYSCFV1
Eco RI
Hind III
Amp R
LEU2
URA3
pCGS515
/SCFV1
10.4Kbp
Bgl II
SCFV1
Sal I
TPI PROMOTER
Hind III
VH
VL
Suc 2 S.S.
Hind III

Fig. 24

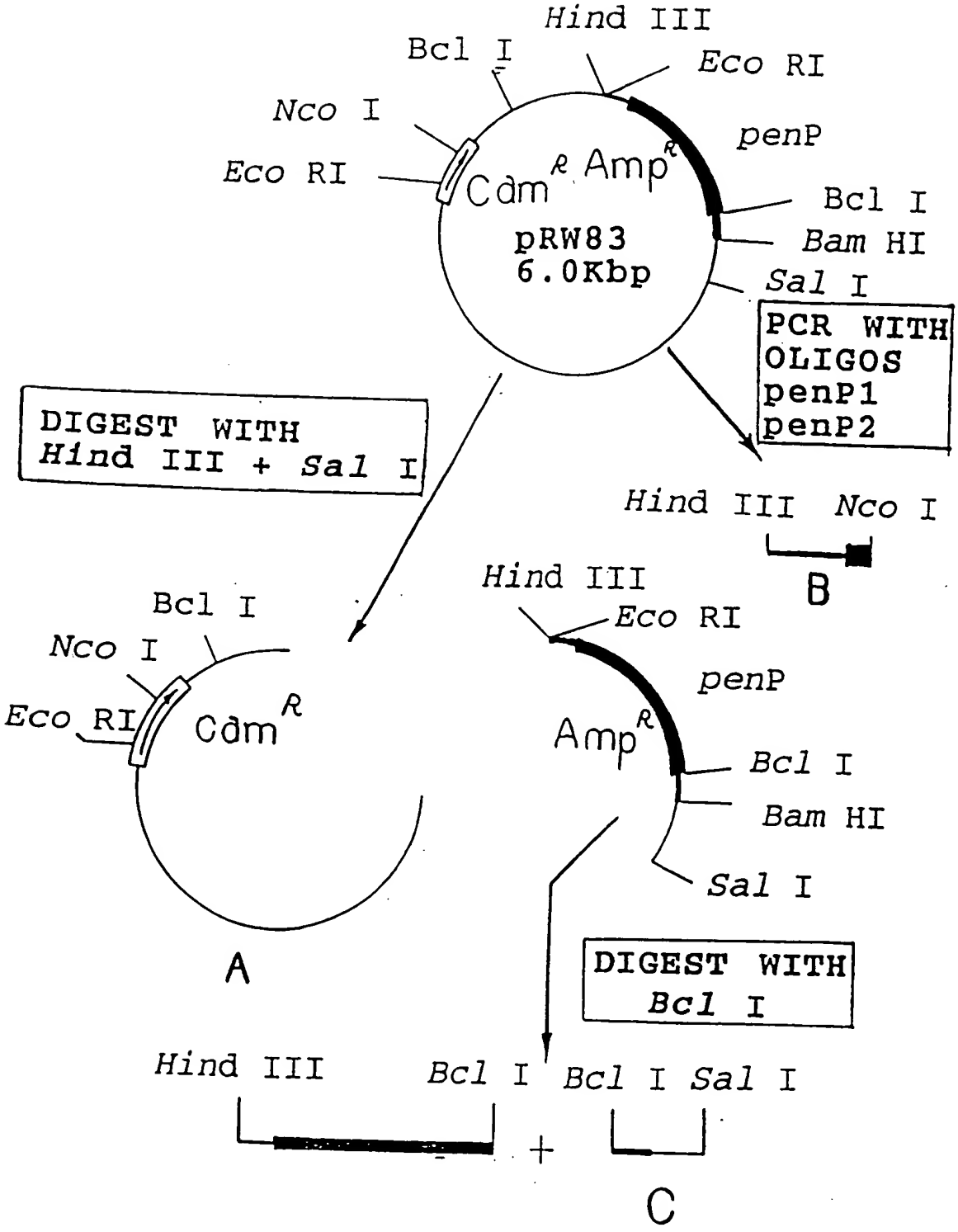
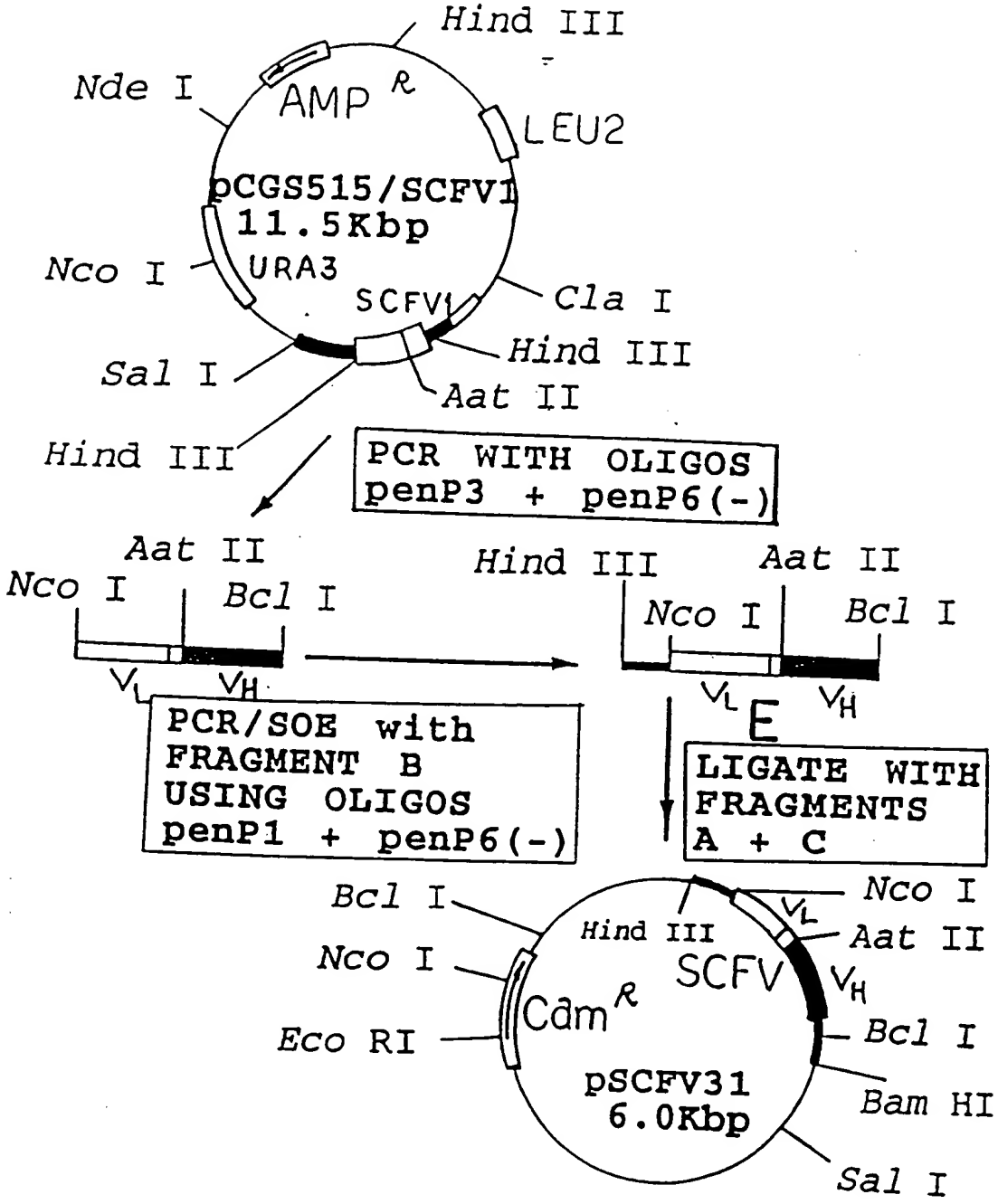

Hind III
Bcl I
Eco RI
Nco I
penP
Eco RI
Cdm^R Amp^R
Bcl I
pRW83
6.0Kbp
Bam HI
Sal I
PCR WITH
OLIGOS
penP1
penP2
DIGEST WITH
Hind III + Sal I
Hind III Nco I
B
Hind III
Bcl I
Eco RI
Nco I
penP
Eco RI
Cdm^R
Amp^R
Bcl I
Bam HI
Sal I
A
DIGEST WITH
Bcl I
Hind III
Bcl I
Bcl I Sal I
+
C

Fig. 24 (CONT.)


Hind III
Nde I
AMP R
LEU2
pCGS515/SCFV1
11.5Kbp
Nco I
URA3
SCFV1
Cla I
Hind III
Sal I
Aat II
Hind III
PCR WITH OLIGOS
penP3 + penP6(-)
Aat II
Nco I
Bcl I
VL
VH
PCR/SOE with
FRAGMENT B
USING OLIGOS
penP1 + penP6(-)
Hind III
Aat II
Nco I
Bcl I
VL
VH
E
LIGATE WITH
FRAGMENTS
A + C
Bcl I
Nco I
Eco RI
Hind III
VL
SCFV
VH
Nco I
Aat II
Cam R
Bcl I
pSCFV31
6.0Kbp
Bam HI
Sal I

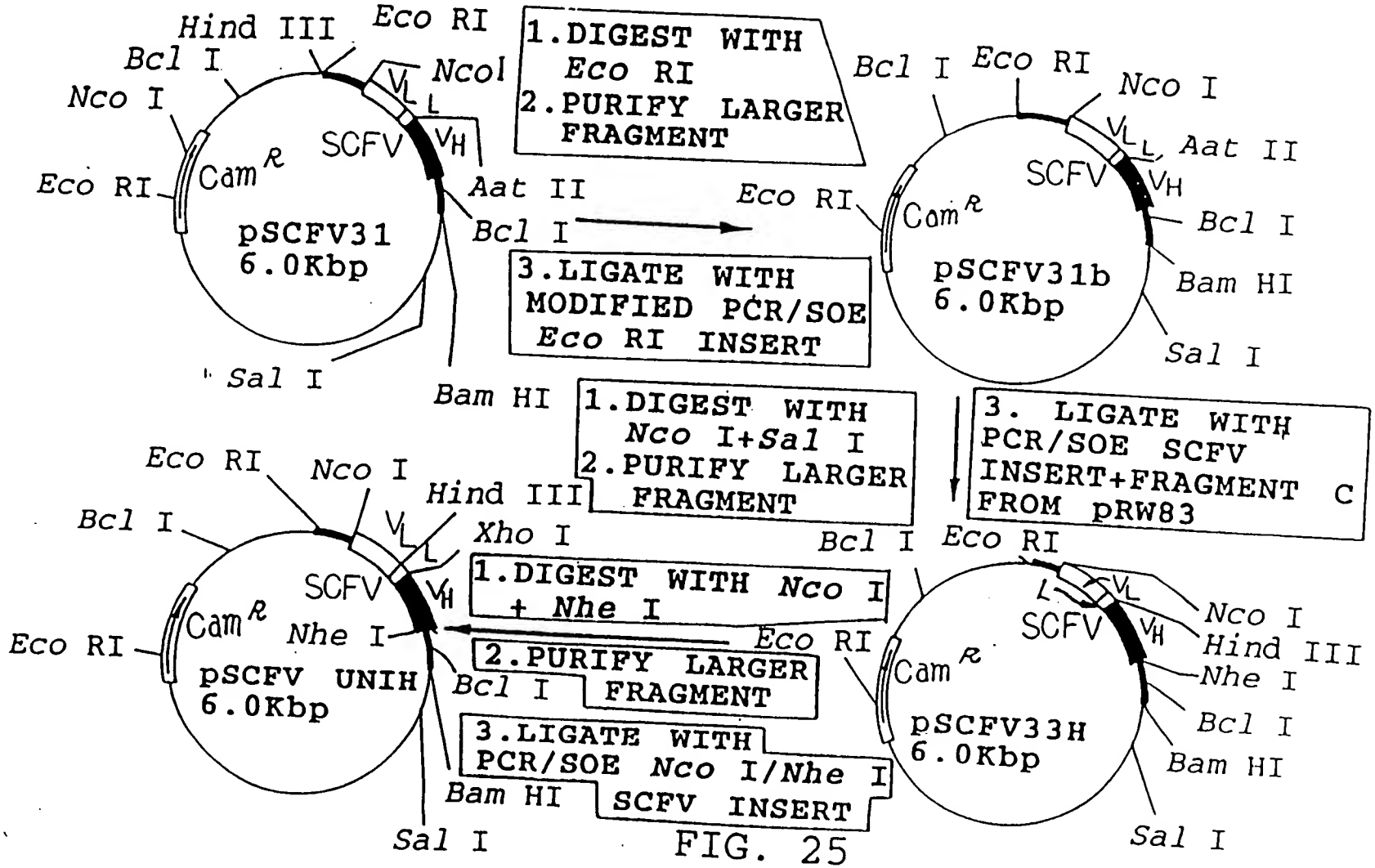

Hind III
Eco RI
Bcl I
NcoI
Nco I
VL
L
SCFV
VH
Eco RI
Cam R
Aat II
pSCFV31
6.0Kbp
Bcl I
Sal I
Bam HI
1.DIGEST WITH Eco RI
2.PURIFY LARGER FRAGMENT
3.LIGATE WITH MODIFIED PCR/SOE Eco RI INSERT
Bcl I
Eco RI
Nco I
VL
L
Aat II
SCFV
VH
Cam R
Bcl I
pSCFV31b
6.0Kbp
Bam HI
Sal I
1.DIGEST WITH Nco I+Sal I
2.PURIFY LARGER FRAGMENT
3. LIGATE WITH PCR/SOE SCFV INSERT+FRAGMENT C FROM pRW83
Eco RI
Nco I
Hind III
Xho I
Bcl I
VL
L
SCFV
VH
Cam R
Nhe I
Eco RI
pSCFV UNIH
6.0Kbp
Bcl I
Sal I
Bam HI
1.DIGEST WITH Nco I + Nhe I
2.PURIFY LARGER FRAGMENT
3.LIGATE WITH PCR/SOE Nco I/Nhe I SCFV INSERT
Bcl I
Eco RI
L
VL
SCFV
VH
Cam R
Eco RI
pSCFV33H
6.0Kbp
Nco I
Hind III
Nhe I
Bcl I
Bam HI
Sal I

FIG. 25

FIG. 26

*Eco* RI

CTCATGTTTG ACAGCTTATC ATCGATGAAT

TCCATCACTT CCCTCCGTTC ATTTGTCCCC

GGTGGAAACG AGGTCATCAT TTCCTTCCGA

AAAAACGGTT GCATTTAAAT CTTACATATG

TAATACTTTC AAAGACTACA TTTGTAAGAT

TTGATGTTTG AGTCGGCTGA AAGATCGTAC

GTACCAATTA TTGTTTCGTG ATTGTTCAAG

CCATAACACT GTAGGGATAG TGGAAAGAGT

GCTTCATCTG GTTACGATCA ATCAAATATT

*pelB* Signal

CAAACGGAGG GAGACGATTT TG | Met Lys Tyr Leu
**Sequence** | ATG AAA TAC CTA

Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA

*Nco* I → | ← $H4V_L$

Leu Ala Ala Gln Pro Ala Met Ala | Asp Ile
CTC GCT GCC CAA CCA GCC ATG GCC | GAC ATC

Val Met Thr Gln Ser Pro Asp Ser Leu Ala
GTG ATG ACC CAG TCT CCA GAC TCC CTG GCT

Val Ser Leu Gly Glu Arg Ala Thr Ile Asn
GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC

Cys Lys Ser Ser Gln Ser Val Leu Tyr Ser
TGC AAG TCC AGC CAG AGT GTT TTA TAC AGC

FIG. 26 (CONT.)

Gln Gln Ser Ala Glu Leu Val Lys Pro Gly
CAG CAG TCT GCT GAG TTG GTG AAA CCT GGG

Ala Ser Val Lys Ile Ser Cys Lys Ala Ser
GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT

Gly Tyr Thr Phe Thr Asp His Ala Ile His
GGC TAC ACC TTC ACT GAC CAT GCA ATT CAC

Trp Val Lys Gln Asn Pro Glu Gln Gly Leu
TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG

Glu Trp Ile Gly Tyr Phe Ser Pro Gly Asn
GAA TGG ATT GGA TAT TTT TCT CCC GGA AAT

Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys
GAT GAT TTT AAA TAC AAT GAG AGG TTC AAG

Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser
GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC

Ser Ser Thr Ala Tyr Val Gln Leu Asn Ser
TCC AGC ACT GCC TAC GTG CAG CTC AAC AGC

Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe
CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC

Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp
TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG

CC49 $V_H$ →

Gly Gln Gly Thr Ser Val Thr Val Ser Ser
GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA

FIG. 26 (CONT.)

*Nhe* I

TAA AAAGCTAGCG ATGAATCCGT CAAAACATCA

*Bcl* I

TCTTACATAA AGTCACTTGG TGATCAAGCT

CATATCATTG TCCGGCAATG GTGTGGGCTT

TTTTTGTTTT CTATCTTTAA AGATCATGTG

AAGGAAAAAA CGGGAAAATC GGTCTGCGGG

AAAGGACCGG GTTTTTGTCG AAATCATAGG

*Bam* HI

CGAATGGGTT GGATTGTGAC AAAATTCGGA TCC

FIG. 27

CONSTRUCTION OF PLASMID pSCFV UHH. GENERATION OF A COMBINATORIAL LIBRARY OF $V_H$ GENES WITH HUM4 $V_L$

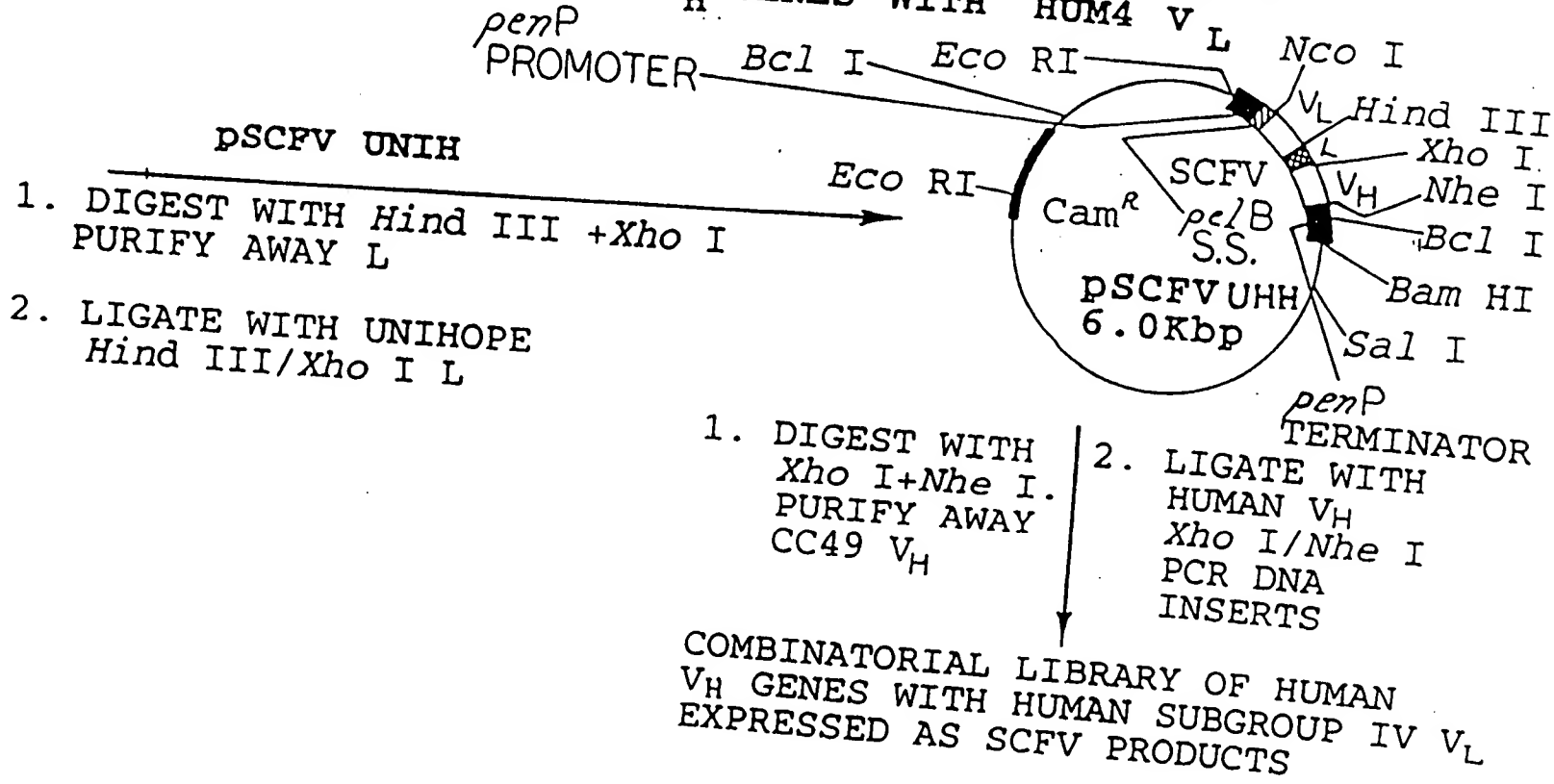


FIG. 28

*Eco* RI

CTCATGTTTG ACAGCTTATC ATCGATGAAT

TCCATCACTT CCCTCCGTTC ATTTGTCCCC

GGTGGAAACG AGGTCATCAT TTCCTTCCGA

AAAAACGGTT GCATTTAAAT CTTACATATG

TAATACTTTC AAAGACTACA TTTGTAAGAT

TTGATGTTTG AGTCGGCTGA AAGATCGTAC

GTACCAATTA TTGTTTCGTG ATTGTTCAAG

CCATAACACT GTAGGGATAG TGGAAAGAGT

GCTTCATCTG GTTACGATCA ATCAAATATT

*pelB* Signal

CAAACGGAGG GAGACGATTT TG|Met Lys Tyr Leu
**Sequence** ATG AAA TAC CTA

Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA

*Nco* I → ← H4V$_L$

Leu Ala Ala Gln Pro Ala Met Ala|Asp Ile
CTC GCT GCC CAA CCA GCC ATG GCC|GAC ATC

Val Met Thr Gln Ser Pro Asp Ser Leu Ala
GTG ATG ACC CAG TCT CCA GAC TCC CTG GCT

Val Ser Leu Gly Glu Arg Ala Thr Ile Asn
GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC

Cys Lys Ser Ser Gln Ser Val Leu Tyr Ser
TGC AAG TCC AGC CAG AGT GTT TTA TAC AGC

FIG. 28 (CONT.)

Ser Asn Asn Lys Asn Tyr Leu Ala Trp Tyr
TCC AAC AAT AAG AAC TAC TTA GCT TGG TAC

Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu
CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG

Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser
CTC ATT TAC TGG GCA TCT ACC CGG GAA TCC

Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC

Ser Leu Gln Ala Glu Asp Val Ala Val Tyr
AGC CTG CAG GCT GAA GAT GTG GCA GTT TAT

Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu
TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC

**$H4V_L$**

Thr Phe Gly Gly Gly Thr Lys Val Val Ile
ACT TTC GGC GGA GGG ACC AAG GTG GTG ATC

***Hind* III LINKER**

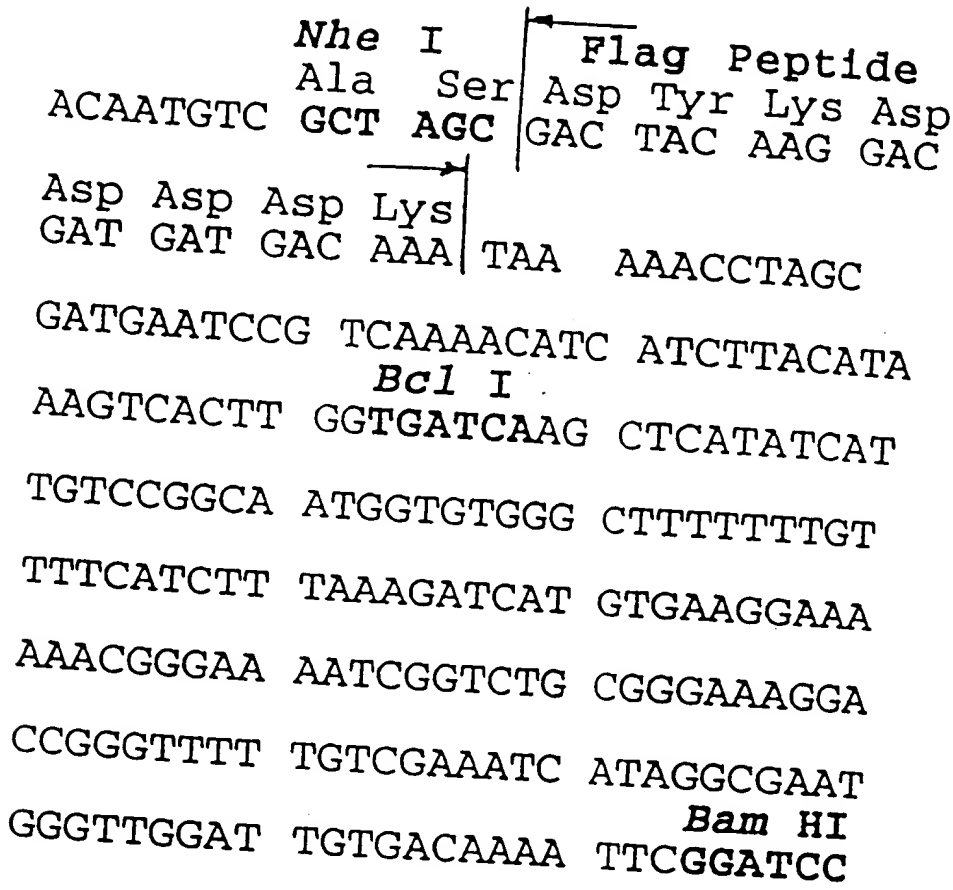
Lys | Leu Ser Ala Asp Asp Ala Lys Lys Asp
**AAG** | **CTT** AGT GCG GAC GAT GCG AAA AAG GAT

Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp
GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC

**LINKER *Xho* I**

Asp Ala Lys Lys Asp Leu | Glu
GAT GCT AAA AAG GAC **CTC** | **GAG**

FIG. 28 (CONT.)


Nhe I
Flag Peptide
Ala Ser
Asp Tyr Lys Asp
ACAATGTC GCT AGC
GAC TAC AAG GAC
Asp Asp Asp Lys
GAT GAT GAC AAA
TAA AAACCTAGC
GATGAATCCG TCAAAACATC ATCTTACATA
Bcl I
AAGTCACTT GGTGATCAAG CTCATATCAT
TGTCCGGCA ATGGTGTGGG CTTTTTTTGT
TTTCATCTT TAAAGATCAT GTGAAGGAAA
AAACGGGAA AATCGGTCTG CGGGAAAGGA
CCGGGTTTT TGTCGAAATC ATAGGCGAAT
Bam HI
GGGTTGGAT TGTGACAAAA TTCGGATCC

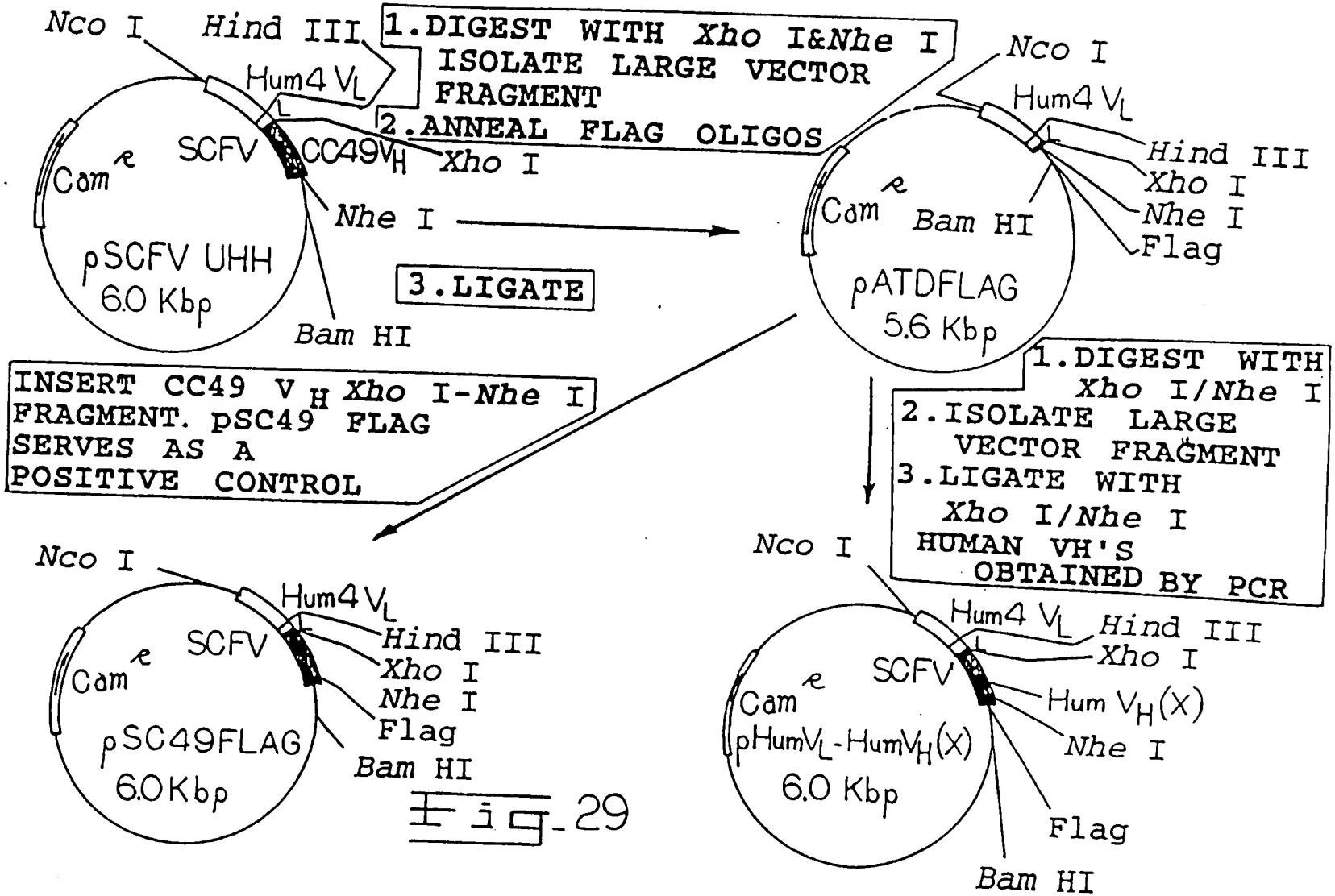

Nco I
Hind III
1.DIGEST WITH Xho I&Nhe I
ISOLATE LARGE VECTOR FRAGMENT
2.ANNEAL FLAG OLIGOS
Hum4 VL
SCFV
CC49VH
Xho I
Nhe I
Cam
pSCFV UHH
6.0 Kbp
Bam HI
3.LIGATE
INSERT CC49 VH Xho I-Nhe I FRAGMENT. pSC49 FLAG SERVES AS A POSITIVE CONTROL
Nco I
Hum4 VL
Hind III
Xho I
Nhe I
Cam
Bam HI
pATDFLAG
5.6 Kbp
Flag
1.DIGEST WITH Xho I/Nhe I
2.ISOLATE LARGE VECTOR FRAGMENT
3.LIGATE WITH Xho I/Nhe I HUMAN VH'S OBTAINED BY PCR
Nco I
Hum4 VL
SCFV
Hind III
Xho I
Nhe I
Flag
Cam
pSC49FLAG
6.0Kbp
Bam HI
Nco I
Hum4 VL
Hind III
Xho I
SCFV
Cam
Hum VH(X)
Nhe I
pHumVL-HumVH(X)
6.0 Kbp
Flag
Bam HI

Fig-29

FIG. 30

*Eco* RI

CTCATGTTTG ACAGCTTATC ATCGATGAAT

TCCATCACTT CCCTCCGTTC ATTTGTCCCC

GGTGGAAACG AGGTCATCAT TTCCTTCCGA

AAAAACGGTT GCATTTAAAT CTTACATATG

TAATACTTTC AAAGACTACA TTTGTAAGAT

TTGATGTTTG AGTCGGCTGA AAGATCGTAC

GTACCAATTA TTGTTTCGTG ATTGTTCAAG

CCATAACACT GTAGGGATAG TGGAAAGAGT

GCTTCATCTG GTTACGATCA ATCAAATATT

*pelB* Signal

CAAACGGAGG GAGACGATTT TG | Met Lys Tyr Leu
ATG AAA TAC CTA

**Sequence**

Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA

*Nco* I → | ← **$H4V_L$**

Leu Ala Ala Gln Pro Ala Met Ala | Asp Ile
CTC GCT GCC CAA CCA GCC ATG GCC | GAC ATC

Val Met Thr Gln Ser Pro Asp Ser Leu Ala
GTG ATG ACC CAG TCT CCA GAC TCC CTG GCT

Val Ser Leu Gly Glu Arg Ala Thr Ile Asn
GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC

Cys Lys Ser Ser Gln Ser Val Leu Tyr Ser
TGC AAG TCC AGC CAG AGT GTT TTA TAC AGC

FIG. 30 (CONT.)

Ser Asn Asn Lys Asn Tyr Leu Ala Trp Tyr
TCC AAC AAT AAG AAC TAC TTA GCT TGG TAC

Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu
CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG

Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser
CTC ATT TAC TGG GCA TCT ACC CGG GAA TCC

Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC

Ser Leu Gln Ala Glu Asp Val Ala Val Tyr
AGC CTG CAG GCT GAA GAT GTG GCA GTT TAT

Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu
TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC

Thr Phe Gly Gly Gly Thr Lys Val Val Ile — **$H4V_L$**
ACT TTC GGC GGA GGG ACC AAG GTG GTG ATC

***Hind* III** **LINKER**

Lys | Leu Ser Ala Asp Asp Ala Lys Lys Asp
**AAG** | **CTT** AGT GCG GAC GAT GCG AAA AAG GAT

Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp
GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC

**LINKER** ***Xho* I** **CC49 $V_H$**

Asp Ala Lys Lys Asp Leu | Glu Val Gln Leu
GAT GCT AAA AAG GAC CTC | GAG GTT CAG TTG

FIG. 30 (CONT.)

Gln Gln Ser Ala Glu Leu Val Lys Pro Gly
CAG CAG TCT GCT GAG TTG GTG AAA CCT GGG

Ala Ser Val Lys Ile Ser Cys Lys Ala Ser
GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT

Gly Tyr Thr Phe Thr Asp His Ala Ile His
GGC TAC ACC TTC ACT GAC CAT GCA ATT CAC

Trp Val Lys Gln Asn Pro Glu Gln Gly Leu
TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG

Glu Trp Ile Gly Tyr Phe Ser Pro Gly Asn
GAA TGG ATT GGA TAT TTT TCT CCC GGA AAT

Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys
GAT GAT TTT AAA TAC AAT GAG AGG TTC AAG

Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser
GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC

Ser Ser Thr Ala Tyr Val Gln Leu Asn Ser
TCC AGC ACT GCC TAC GTG CAG CTC AAC AGC

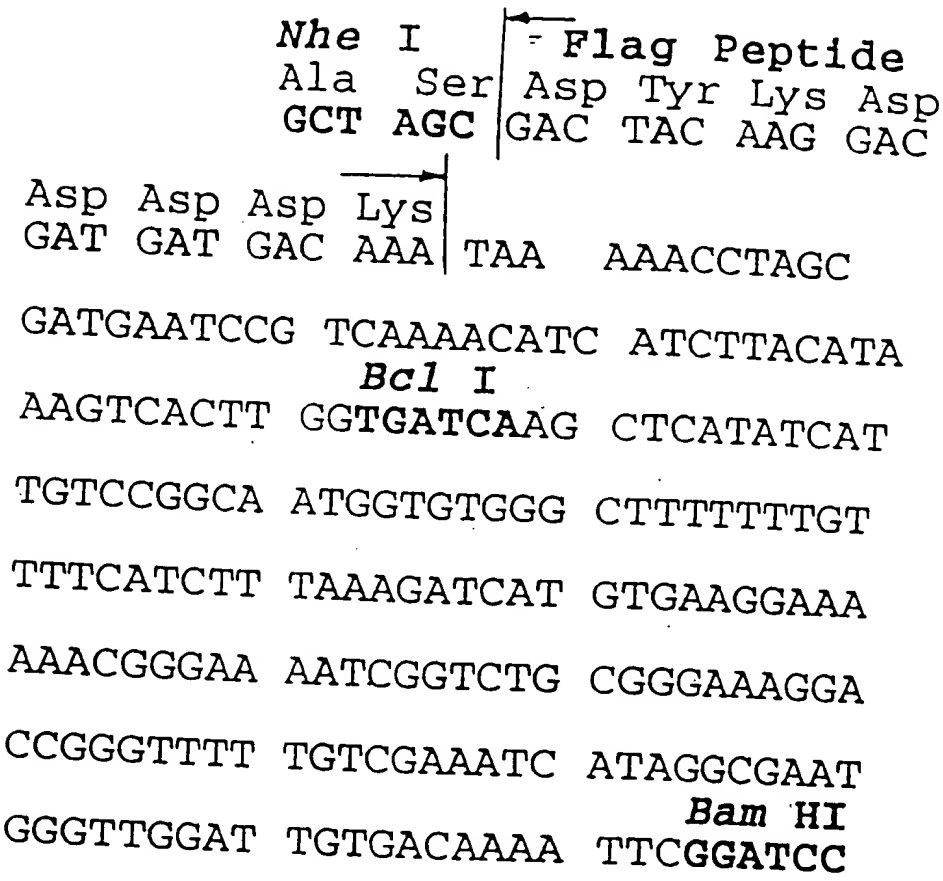
Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe
CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC

Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp
TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG

**CC49 $V_H$** →

Gly Gln Gly Thr Ser Val Thr Val Ser Ser
GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA

FIG. 30 (CONT.)


*Nhe* I
Flag Peptide
Ala Ser Asp Tyr Lys Asp
GCT AGC GAC TAC AAG GAC
Asp Asp Asp Lys
GAT GAT GAC AAA TAA AAACCTAGC
GATGAATCCG TCAAAACATC ATCTTACATA
*Bcl* I
AAGTCACTT GGTGATCAAG CTCATATCAT
TGTCCGGCA ATGGTGTGGG CTTTTTTTGT
TTTCATCTT TAAAGATCAT GTGAAGGAAA
AAACGGGAA AATCGGTCTG CGGGAAAGGA
CCGGGTTTT TGTCGAAATC ATAGGCGAAT
*Bam* HI
GGGTTGGAT TGTGACAAAA TTCGGATCC

# Figure 31

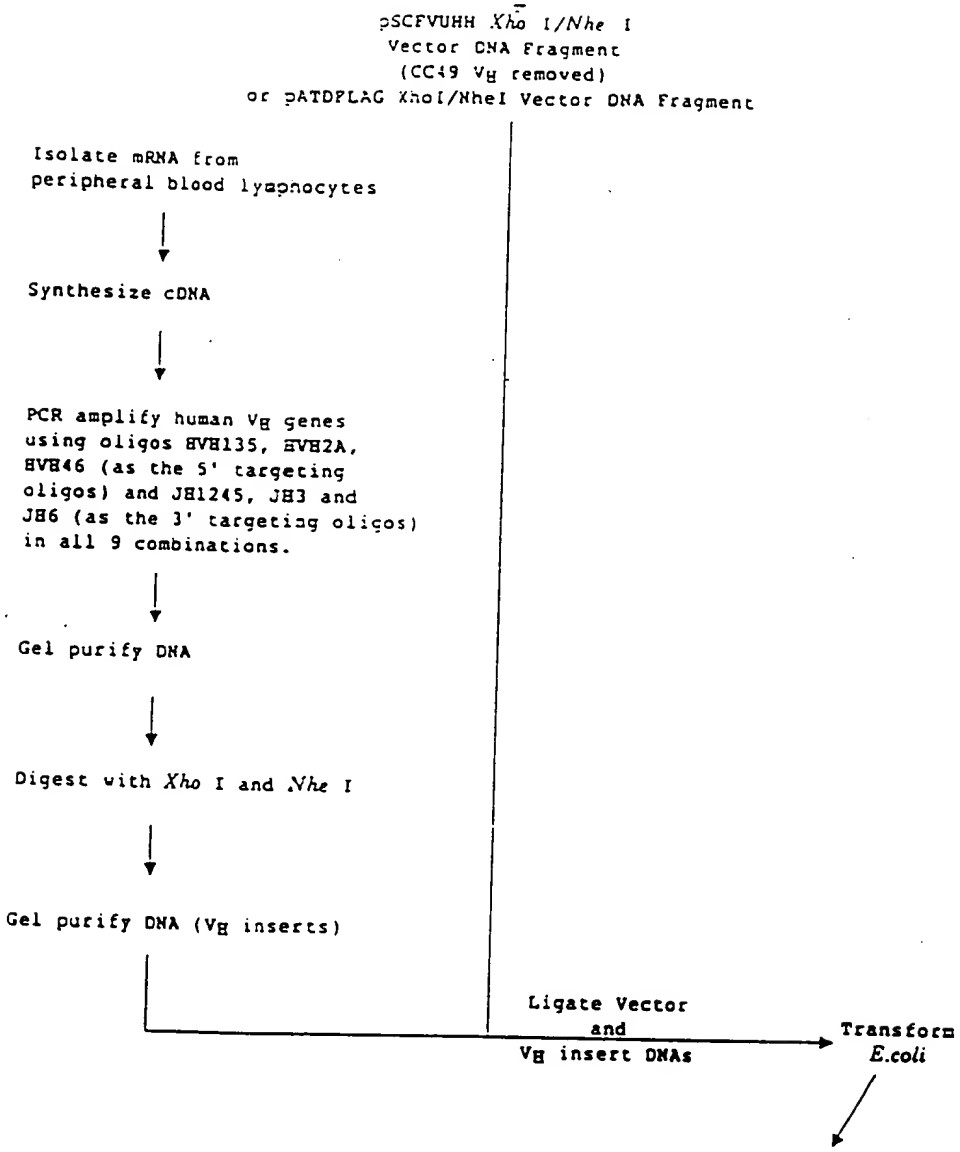

pSCFVUHH Xho I/Nhe I
Vector DNA Fragment
(CC49 VH removed)
or pATDFLAG XhoI/NheI Vector DNA Fragment
Isolate mRNA from peripheral blood lymphocytes
Synthesize cDNA
PCR amplify human VH genes using oligos HVH135, HVH2A, HVH46 (as the 5' targeting oligos) and JH1245, JH3 and JH6 (as the 3' targeting oligos) in all 9 combinations.
Gel purify DNA
Digest with Xho I and Nhe I
Gel purify DNA (VH inserts)
Ligate Vector and VH insert DNAs
Transform E.coli

Plate transformation mix onto hydrophilic membranes (137 mm) which are placed on LB CAM 20 agar plates (150 mm) with a colony density of ≤ 50,000 per plate. Grow for 8-16 hours at 37 °C.

SCFV is secreted by *E.coli* and may bind to TAG.

Transfer hydrophilic membrane onto fresh LB CAM 20 plate having a TAG-72-coated hydrophobic membrane (137 mm) already placed on the agar surface. Incubate for 24-96 hours.

assay

Process hydrophobic membrane using a prototype biotinylated TAG-competing antibody, e.g. B72.3, CC49, CC83 or biotinylated competing peptide or mimetic. Use streptavidin conjugated with alkaline phosphatase to bind to biotin and suitable substrate for alkaline phosphatase to develop a color reaction.

Co-relate clear zones on membrane assay with colony(ies) on hydrophilic membrane. Isolate/purify correct clone as necessary. Characterize DNA (sequence) and determine binding affinity of SCFV to TAG-72. Purify SCFV and perform *in vivo* animal biodistribution studies.

Determine normal:tumor tissue binding profile by immunohistochemistry.

Utilize Eum4 $V_L$ and $V_H$ in preferred antibody formats e.g. whole Ig (IgG1, IgE, IgM etc.) Fab or $F(ab')_2$ fragment, or SCFV.

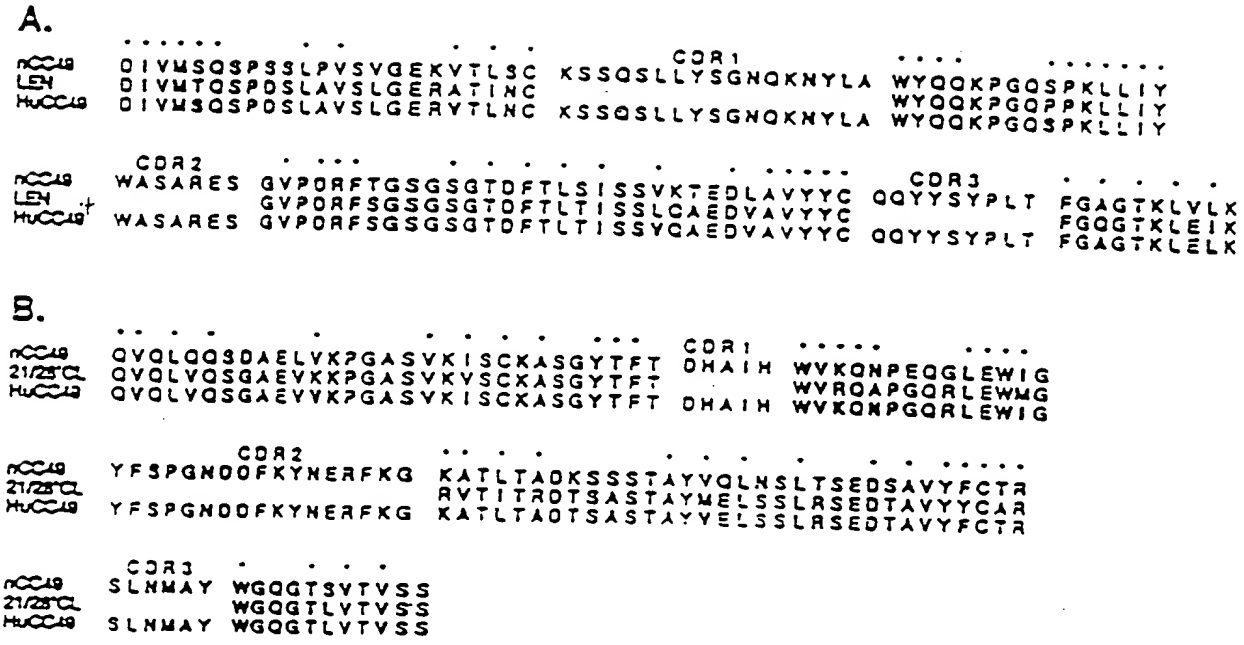



FIG. 32: "Humanization" protocols for MAb CC49. (A) Amino acid sequence of nCC49 $V_L$, $V_L$ frameworks of human MA LEN, and humanized $V_L$ of CC49 (HuCC49) derived from nCC49 and LEN. (B) Amino acid sequence of nCC49 $V_H$, $V_H$ frame works of human MAb 21/28'CL and humanized $V_H$ of CC49 (HuCC49) derived from nCC49 and 21/28'CL MAbs. Framewor residues that are deemed to be important in maintaining the combining-site structure of CC49 on the basis of the known struc ture of the antigen-binding regions of McPC603 (for $V_L$) and 36–71 (for $V_H$) are marked by an asterisk. Framework residues tha are different in CC49 and in the template human sequences and that were retained in the humanized version are highlighted. Th template human sequences were chosen on the basis of greatest similarity with CC49 in the residues that are deemed to be im portant in maintaining the combining-site structure.

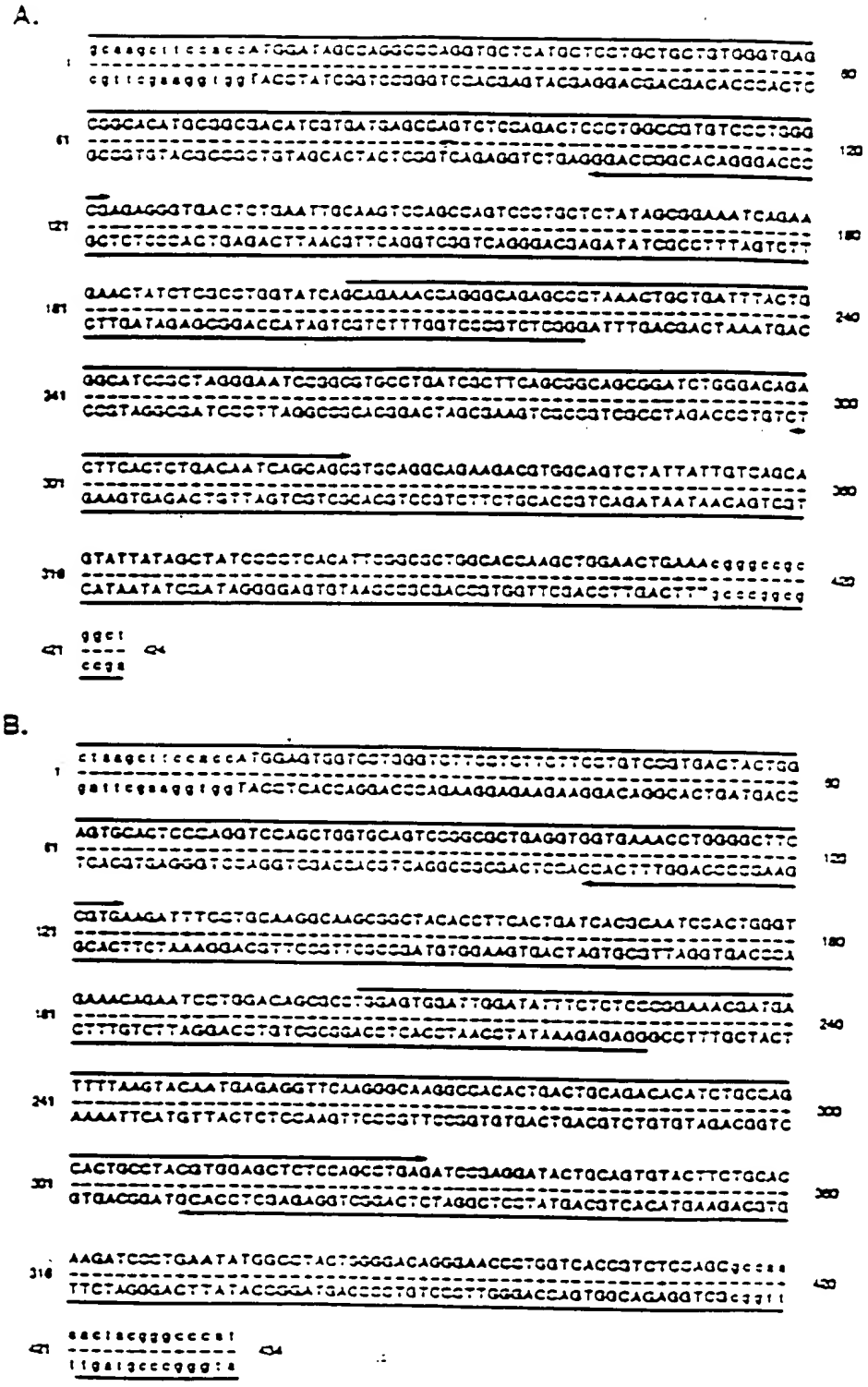



FIG. 33. Nucleotide sequence of the humanized CC49 (HuCC49) variable region genes. Nucleotide sequence of the humanized $V_L$ (A) and $V_H$ (B) genes that were generated and amplified by PCR using four overlapping synthetic oligonucleotides (indicated by arrows) that together encompass, on alternating strands, the entire sequence of each of the genes and its leader. Sequences of the flanking oligomers that do not encode the variable region domains or their leader peptides are shown by lowercase letters. The $V_L$ region (A) is encoded by nucleotides from position 74 to 412, while nucleotides from position 70 to 415 in (B) comprise the $V_H$ region.

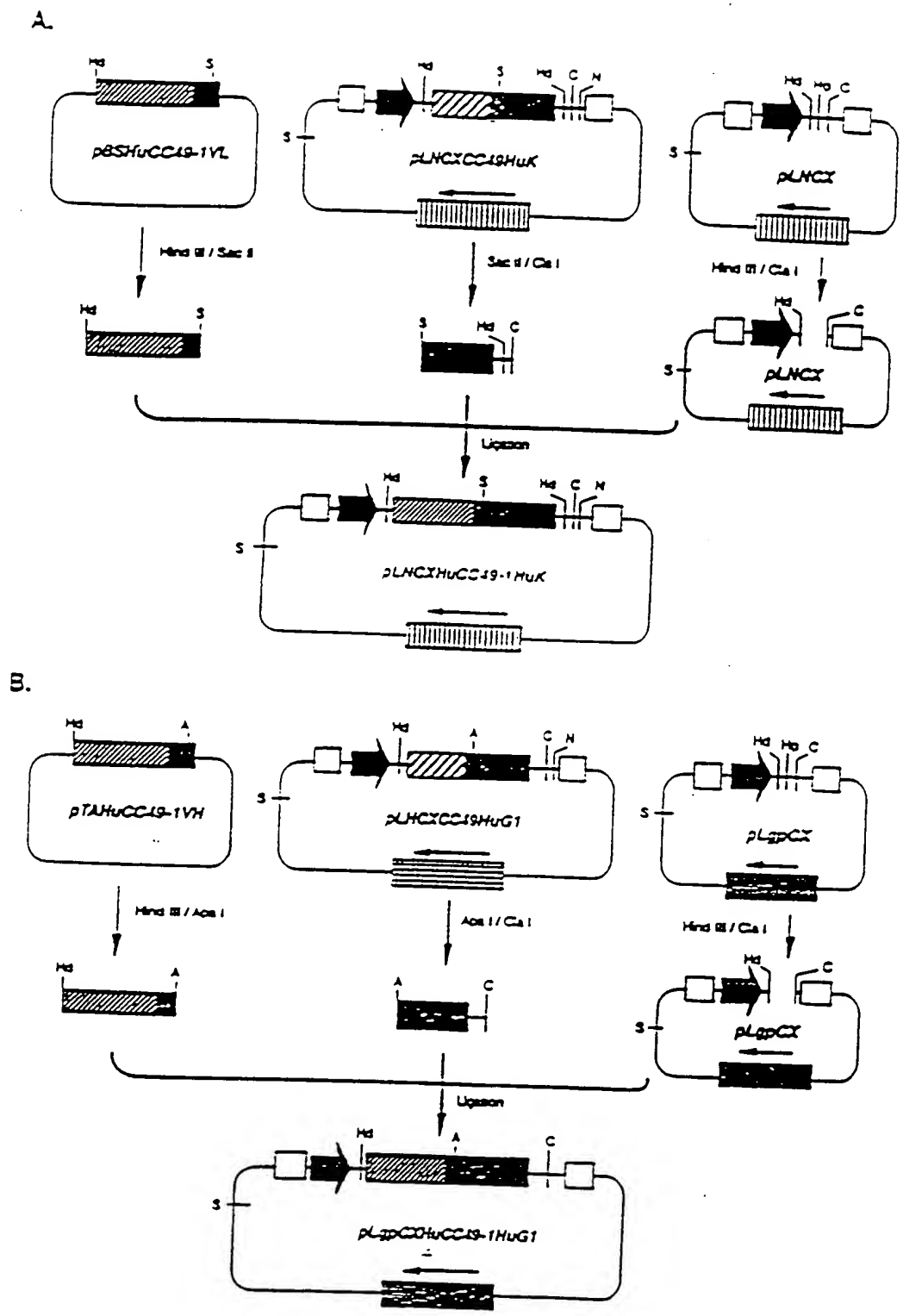



FIG. 34. Schematic representation of the eukaryotic expression constructs of the humanized light (A) and heavy (B) chains of HuCC49. Thin lines represent sequences derived from the prokaryotic vectors pBR322, pBluescript SK⁻, or pCR II. Thick lines depict human κ or γ1 constant regions. Densely hatched boxes represent the humanized variable region, while sparsely hatched boxes indicate murine variable regions. Boxes with vertical, horizontal, or cross bars show neomycin, mycophenolic acid, or hygromycin resistance genes; thin arrows show their transcriptional direction. Empty boxes [illegible]

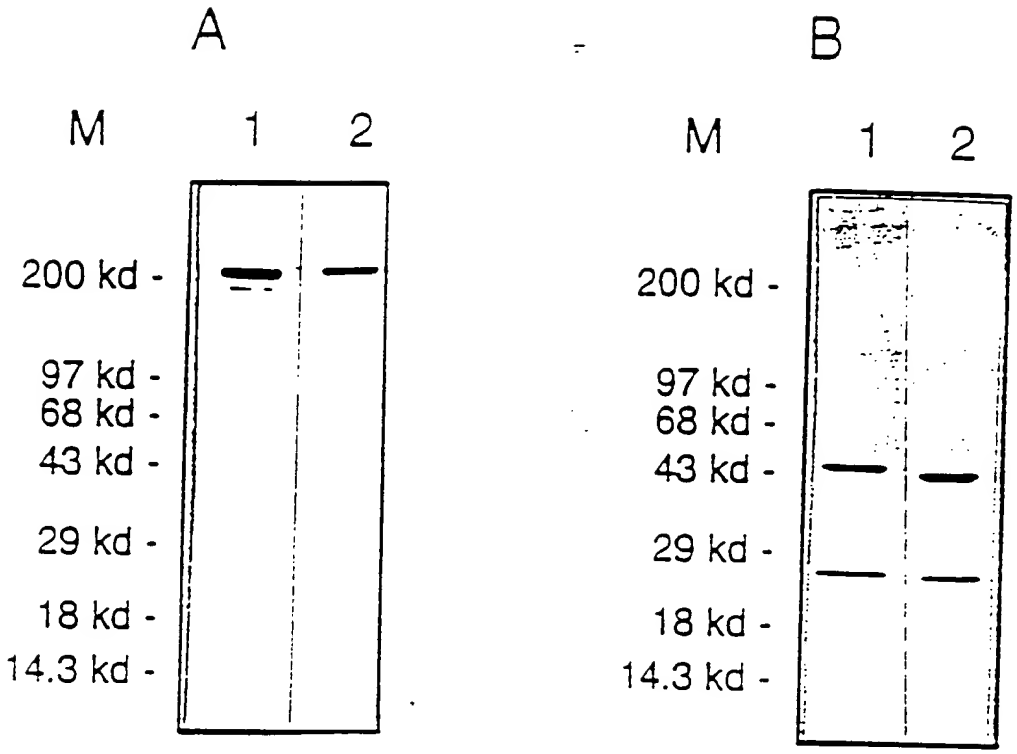



FIG. 35. SDS-PAGE analysis of purified HuCC49 and cCC49. (A) Nonreduced samples on SDS-PAGE. (B) Reduced samples on SDS-PAGE. M, molecular weight markers; lane 1, cCC49; lane 2, HuCC49.

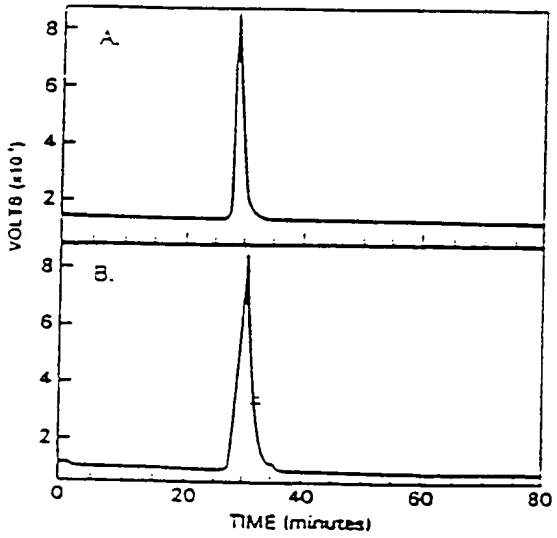



FIG. 36. HPLC analysis of radioiodinated HuCC49 and cCC49 MAbs. $^{131}$I-labeled HuCC49 (A) was compared to $^{125}$I-labeled

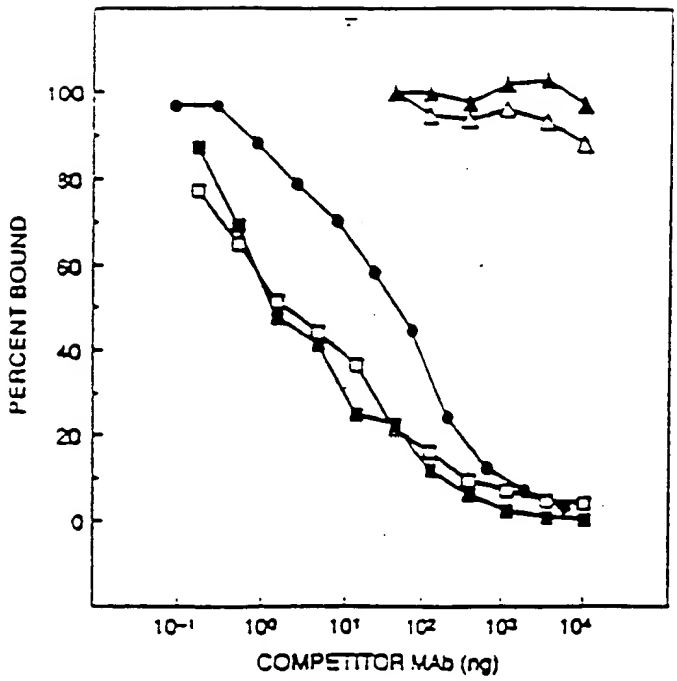



FIG. 37. Reactivity of HuCC49, cCC49, and nCC49 in a competition RIA. MAbs HuCC49 (closed circles), cCC49 (open squares), and nCC49 (closed squares) were used at increasing concentrations to compete for the binding of $^{125}$I-labeled nCC49 to the TAG-72-positive BSM. Controls: Human IgG (closed triangles) and MOPC-21 (open triangles).

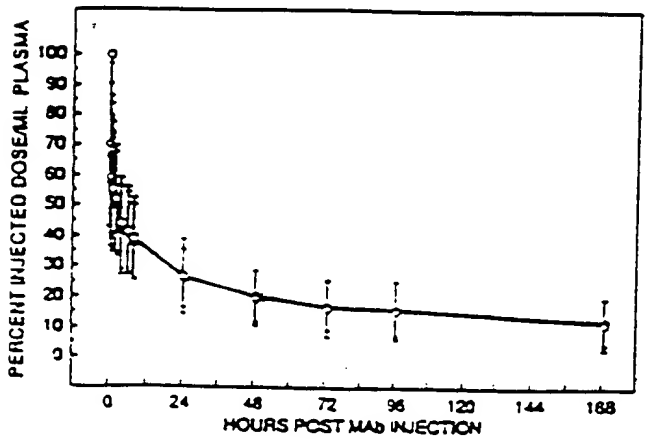



FIG. 38. Clearance of radioiodinated HuCC49 and cCC49 MAbs from the serum of mice. A mixture containing $^{131}$I-la-